THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXI DECEMBER, 1940 No. 6

INDOLE AS A PRECURSOR IN THE SYNTHESIS OF TRYPTOPHAN BY BACTERIA.

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Received for publication September 2, 1940.

FILDES, Gladstone and Knight (1933) stated that *Bact. typhosum* ordinarily would not grow unless tryptophan was present in the nutrients, but that it could be trained to grow without tryptophan which was then synthesized. Fildes and Knight (1933) found the same relationship to tryptophan and growth with a number of other bacteria.

Many instances are known of the synthesis of essential metabolites by bacteria which can grow in their absence, and it is generally accepted that the need for an essential metabolite in the nutrients is due to an inability of a bacterium to synthesize it. This view does not differ from that held in connection with vitamins in animal nutrition or essential foodstuffs in other fields of biology.

It has been pointed out (*cf.* for instance Fildes, 1940) that the synthesis of a nitrogenous essential metabolite from the simplest source of nitrogen, ammonia, probably proceeds in a series of stages. The evidence on which this view is based is indirect, and depends upon the fact that a complex essential metabolite which is required in the nutrients of some bacteria need not be given to others, but only some fraction of it. It is assumed that the final product present in the cells is synthesized by the different bacteria from the less complex substances which are thus stages in the synthesis.

In the case of cozymase the hypothetical stages of synthesis may be represented thus :

$$NH_3 \text{ or Amino-N} \longrightarrow \text{Nicotinic acid} \longrightarrow \text{Cozymase.}$$

The existence of these stages is deduced from the fact that the finished product, cozymase, is required for the growth of *H. influenzae*, whereas

nicotinic acid is adequate for *Proteus* and amino-N for *Bact. typhosum*. *Bact. coli* is satisfied with the basic substance ammonia.

Although it is assumed for schematic purposes that nicotinic acid is actually a stage in the synthesis, it is admitted that nicotinic acid may act only because it is readily converted by the bacterium into another related substance which is the real stage in the synthesis.

Stages in the synthesis of other essential metabolites can be deduced on similar grounds, but no attempt has been made to analyse the synthesis of tryptophan. Preliminary experiments were carried out to test whether *Bact. typhosum*, reputed to require tryptophan for growth, could in fact grow with some related but less complex substance. The following were tested :

Indoleacrylic acid, indoleacetic acid, indolepropionic acid, indolecarboxylic acid, indolepyruvic acid, indole ethylamine, indole aldehyde, skatole and indole.

It was found that indole itself was highly active as a growth factor, and a trace of growth was present in indolecarboxylic acid which, however, contained indole as an impurity. The other substances were entirely inactive. This observation suggested that indole, or possibly some unknown but similar substance, was in fact a stage in the synthesis of tryptophan by *Bact. typhosum* according to the following scheme :

$$NH_3 \text{ or Amino-N} \longrightarrow \text{Indole} \longrightarrow \text{Tryptophan.}$$

If this scheme is correct it should be possible to find bacteria which can grow, i.e. synthesize tryptophan, when supplied with any of these stages.

A number of bacteria are known which grow when ammonia is the sole source of nitrogen, and these may be taken as examples of those which can achieve all stages of the synthesis, i.e. *Bact. coli*, or *Bact. typhosum*, after training (Fildes, Gladstone and Knight, 1933). It is also known that strains of *B. anthracis* grow when amino-N without tryptophan is supplied (Gladstone, 1939), and that the same applies to staphylococcus (Gladstone, 1937). These strains are thus examples of bacteria which can synthesize tryptophan from amino-N. On the other hand it is recorded that a number of strains of different bacteria grow only in the presence of tryptophan, without the stages amino-N $\longrightarrow$ tryptophan having been analysed, namely, *Bact. typhosum* (Fildes, Gladstone and Knight, 1933), *Cl. sporogenes*, *Cl. botulinum*, *C. diphtheriae*, *B. anthracis*, and staphylococcus (Fildes and Knight, 1933). In the present work this analysis is attempted.

PROCEDURE.

Several strains of bacteria were selected which were known to require tryptophan for growth. These were inoculated into a nutrient mixture which was adequate for growth only when tryptophan was added. Serial dilutions of tryptophan and indole were then supplied and the result noted. The general technique was as described by Gladstone (1939), but in all cases the number of organisms added per 10 ml. tube was approximately 1000–2000.

For *Bact. typhosum* the basal medium was the amino-acid mixture mentioned by Gladstone without, however, Mg or Fe. Glucose was added as a source of

energy. For staphylococcus the same mixture was used *plus* nicotinamide to $10^{-5}M$. and aneurin to $10^{-7}M$. For *C. diphtheriae* there was a further addition of pimelic acid to $10^{-4}M$. and an impure preparation of pantothenic acid which was free from tryptophan.

The following strains were used:

Bact. typhosum—Ty 2, O901, H901 from Dr. Felix, and GW1 from Dr. Gleeson White.

C. diphtheriae—Nos. 7 and 13, both *mitis*, from Prof. H. D. Wright, and 04 (*mitis*) from Dr. V. Glass.

Staphylococcus—Nos. 7, 11, 17, 18 of Gladstone (1937).

The inoculum was collected from an agar slope and suspended and diluted in the basal medium.

RESULTS.

Exp. 1 gives an example of the effect of tryptophan and indole in the growth of *Bact. typhosum*. It is seen that growth takes place with indole, though in this case some ten times the concentration adequate for tryptophan is necessary.

Other bacteria were tested in the same way, and Exp. 2 shows the readings at three days.

It is seen that the four strains of *Bact. typhosum* and the three strains of *C. diphtheriae* grow with indole in a high dilution, although usually somewhat more indole is required than is adequate with tryptophan. In the case of staphylococcus, one strain, No. 18, could not use indole in place of tryptophan, while the other three showed increasing capacity to use indole. Staphylococcus No. 18 is therefore in the same position to tryptophan as is *H. influenzae* to cozymase, while the other strains are in the position of *Proteus* to nicotinic acid, and it is concluded (1) that none of the strains can synthesize indole from amino-N; (2) that strains 7, 11 and 17 can synthesize tryptophan from indole; and (3) that strain 18 cannot.

Further, since out of 25 strains examined by Gladstone (1937) 21 grew without tryptophan (or indole), it must be concluded that most strains can synthesize indole and tryptophan from amino-N, and indeed that Gladstone's "trained" strain can synthesize all stages from ammonia.

EXP. 1.—*The Effect of Indole on the Growth of* Bact. typhosum.

Each tube contained the basal amino-acid mixture (7 ml.). The tryptophan and indole were sterilized (indole by filtration) as $10^{-3}M$. solutions, 1 ml. and dilutions being added or water to balance. Each tube then received 2 ml. of a mixture containing per 2 ml.—water 0·43, buffer 0·9, $M/2$ glucose 0·25, $M/200$ cystine in N/10 HCl 0·2, N/1 NaOH 0·02, $M/100$ methionine 0·1, bacteria 2000 in 0·1 ml. The tubes were poured into 50 ml. conical flasks and incubated in air *plus* 5 per cent. CO_2.

Flask.	Medium *plus*.	Molarity.	Growth in hours and days. 22.	54.	3.
1	0	..	0	0	0
2	Tryptophan	10^{-3}	+++	+++	+++
3	,,	10^{-4}	+++	+++	+++
4	,,	10^{-5}	++	++	++
5	,,	10^{-6}	tr.	±	+
6	,,	10^{-7}	0	0	0
7	Indole	10^{-3}	++	+++	+++
8	,,	10^{-4}	+	+	+
9	,,	10^{-5}	0	tr.	tr.
10	,,	10^{-6}	0	0	0
11	,,	10^{-7}	0	0	0

Plus signs in proportion to growth. tr. = trace.

EXP. 2.—*The Growth of Various Bacteria with Tryptophan and Indole.*

		Bact. typhosum.				*C. diphtheriae.*			Staphylococcus.			
		Ty 2.	O 901.	H 901.	G.W. 1.	7.	04.	13.	18.	17.	7.	11.
No addition		0	0	0	0	0	0	0	0	0	0	0
Plus												
Tryptophan	10^{-4}M.	+	+	+	+	+	+	+	+	+	+	+
,,	10^{-5}	+	+	+	+	+	+	+	+	+	+	+
,,	10^{-6}	+	+	+	+	+	+	+	+	+	+	+
,,	10^{-7}	0	0	0	+	0	0	0	0	0	+	0
Indole	10^{-4}M.	+	+	+	+	+	+	+	0	+	+	+
,,	10^{-5}	+	+	+	+	+	+	+	0	0	+	+
,,	10^{-6}	0	+	+	+	0	0	+	0	0	0	+
,,	10^{-7}	0	0	0	0	0	0	0	0	0	0	0

+ Indicates visible growth on the third day of incubation.

The species staphylococcus thus presents instances of failure to synthesize tryptophan and all grades of ability to synthesize it.

In the case of *Bact. typhosum* no normal strains have been described which can grow without tryptophan, but the strain of Fildes, Gladstone and Knight (1933) "Rawlings" was trained to grow without this substance. The normal "Rawlings" strain grows readily with indole, and therefore it must be assumed that its normal disability is a failure to synthesize indole, and that it is this disability (among others) which is overcome by training.

DISCUSSION.

The experiments show that a number of bacteria grow, i.e. synthesize tryptophan, when supplied with nutrients which contain precursors of tryptophan, whereas others require the finished product. There is thus experimental basis for the hypothesis that tryptophan is synthesized from ammonia in stages, and that one of these is indole or some closely related substance. It is also clear that the alteration of synthetic ability which is induced by training consists, in part at least, of the development of power to synthesize indole.

The generally-accepted conclusion that failure to grow is due to failure to synthesize is supported. Burrows (1939), on the other hand, claims to have shown that a strain of *Bact. typhosum* which required tryptophan to start growth synthesized not only tryptophan, but also indole during growth on ammonia, and considers the assumption fallacious that the requirement of tryptophan for growth implies failure to synthesize.

It may, however, be pointed out that the hypothesis is applicable only in general terms. No doubt all bacteria potentially are capable of synthesizing tryptophan, but under the conditions of the experiment the degree of synthesis is inadequate for growth. The fact that by a manipulation of their nutrients they may easily be altered to grow without tryptophan indicates that their potential capacity for synthesis can be easily increased, and indeed variable powers of growth without tryptophan are common in nature within one species.

The hypothesis goes no farther than to suggest that a bacterium does not grow in the absence of a certain essential metabolite because it cannot synthesize it adequately under the test conditions.

CONCLUSIONS.

(1) Certain bacteria which have been shown to require tryptophan for growth are capable of using indole.

(2) It is deduced that tryptophan is synthesized by bacteria from ammonia in stages, one of which is indole.

(3) The hypothesis that failure to grow is due to failure to synthesize is supported.

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THE CONCENTRATION AND DETOXIFICATION OF HUMAN URINE FOR BIOLOGICAL PREGNANCY DIAGNOSIS.

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Received for publication October 16, 1940.

ALTHOUGH several methods for the preparation of gonadotropic hormone extracts from human urine for application to the biological pregnancy tests are available, all methods without exception require a great deal of patience and perseverance before they can be made to furnish a consistent product free from toxic substances and suitable for application to the Zondek-Aschheim, Friedman and Hogben (*Xenopus laevis* toad) tests. Even after considerable experience of the conditions underlying the concentration and detoxification of septic urines with alcohol, acetone, or ether, the preparation is always liable to go astray despite the most careful handling, with the consequent death of several test animals and the ruination of many tests.

The method which is about to be described is one which has obvious advantages over existing methods, in that, while its steps are the simplest and its reagents inexpensive and readily available, it yields an extract with a precision and certainty unequalled by any of the procedures at present in use.

METHOD.

The steps in the method are :

1. The adsorption of the gonadotropic hormone from the urine with kaolin at pH 4·0, using brom-phenol blue as indicator.

2. The removal of the kaolin suspension by centrifugalization, and the extraction of the hormone from the kaolin precipitate by means of N/10 sodium hydroxide, the alkaline suspension being again centrifuged, and the supernatant fluid finally adjusted to a faintly acid reaction to litmus, when it is ready for injection into the test animal.

Reagents and Apparatus.

1. Brom-phenol blue solution. 0·4 g. of solid is ground up with 15 c.c. of N/20 sodium hydroxide in a small mortar, and when completely dissolved the volume is diluted to 100 c.c. with water.

2. 10 per cent. and 20 per cent. (by volume) hydrochloric acid.

3. 20 per cent. aqueous suspension of kaolin (kaolin washed with acids, B.D.H.).

4. N/10,000 hydrochloric acid (pH 4·0). 0·1 c.c. of normal hydrochloric acid diluted to 1000 c.c. with water.

5. N/10 sodium hydroxide, CO_2-free. Prepared freshly each day by adding 0·5 c.c. of 40 per cent. sodium hydroxide to 50 c.c. of water.
6. Solid anhydrous dextrose, B.D.H.
7. Absolute alcohol.
8. 100 c.c. and 250 c.c. graduated cylinders.

Technique.

Standard technique for the Friedman rabbit test.

Into a 100 c.c. graduated cylinder introduce 25 c.c. of urine, add 25 c.c. of water and 0·25 c.c. of brom-phenol blue indicator. With dilute samples it is necessary to use 50 c.c. of urine, omitting the 25 c.c. of water as above. Adjust the reaction to pH 4·0 by the addition of 20 per cent. hydrochloric acid, adding about 0·25 c.c. of acid at a time, with constant shaking until the colour changes from deep blue to a point where the blue almost disappears—a faint bluish-green coloration superimposed on the yellowish background of the urine itself.* 5 c.c. of well-shaken suspension of kaolin is added and the urine thoroughly mixed by inversion several times. After allowing the clay to flocculate and settle to a layer below about the 30 c.c. mark, the supernatant fluid is carefully poured off, the kaolin suspension transferred to a round-bottomed centrifuge tube of 50 c.c. capacity, and centrifuged at 2000 to 3000 r.p.m. for two to three minutes. If the urine has undergone bacterial decomposition the precipitate is ground up and washed with 40 to 50 c.c. of N/10,000 hydrochloric acid and again centrifuged.

The supernatant fluid is decanted and the kaolin precipitate thoroughly ground up into 10 c.c. of the N/10 sodium hydroxide by means of a thick-walled test tube. The emulsion should be homogeneous and free from all lumps. The mixture is centrifuged for 5 to 10 minutes at 2000 to 3000 r.p.m., after which the supernatant fluid (10 c.c. in volume) containing the gonadotropic hormone is made slightly acid to litmus (approximately pH 5·5 to 6·0) by the addition of 10 per cent. hydrochloric acid, drop by drop. Usually about 0·75 of 10 per cent. hydrochloric is required to render the extract slightly acid. 0·5 c.c. of absolute alcohol is now added, followed by 1–2 g. of solid glucose. After warming to 37° C. to dissolve the glucose, the extract is ready for immediate injection, or, on the other hand, the extract is stable for several weeks if stored in the refrigerator.

The ovaries of the rabbit are examined at the usual 48 hours' interval after injection.

For the Zondek-Aschheim Mouse Test:

0·25 c.c of the above extract is injected twice daily for two days, and the ovaries examined on the fifth day.

For the Hogben Toad Test:

With this test the importance of using a highly concentrated extract of urine cannot be over-emphasized (Crew, 1939), as the test depends largely on

* It is important to note that the indicator is dichroic, and therefore the observations on the colour change should be made from a source of light passing horizontally through the standard cylinder.

the quantitative considerations of mass action of the gonadotropic hormone on the ovaries of the toad. Slight alteration is therefore necessary with the standard extraction technique and the essential difference can be summarized as follows :

To 100 c.c. of urine in a 250 c.c. graduated cylinder add 100 c.c. of water. Add 1 c.c. of brom-phenol blue indicator, and after mixing well, adjust the reaction to pH 4·0 by the addition of 20 per cent. hydrochloric acid. 10 c.c. of kaolin is added and the standard method then followed in detail. The trace of alcohol should be omitted from this extract, as its presence is not well tolerated by the toads. The final acidified extract may sometimes be slightly opalescent due to the high degree of concentration, but this in no way interferes with the subsequent biological reaction.

Four toads are employed for the test, each animal being injected intraperitoneally with 2·5 c.c. of extract (equivalent to 25 c.c. of urine), the bulging sides of the toad making this route of injection much easier and safer than the dorsal lymph-sac technique recommended by Bellerby (1934, 1935). A positive reaction is denoted by the appearance of ova in the test jar between a period of from 5 to 30 hours after injection, and a reddening of the toad's cloaca which precedes and is present during the expulsion of ova.

A combined Friedman and Hogben test can be performed simultaneously by injecting three toads and one rabbit each with 2·5 c.c. of extract, this procedure having the advantage of obtaining a result from the toads within a few hours if the reaction is positive, which in certain cases is important from the psychological or the gynaecological standpoint. It also allows the rabbit to be retained and put aside from 4 to 6 weeks, when the animal is in a fit state to be used again for further tests. Moreover, with very early pregnancies (period approximately one week overdue), or with cases where the hormonal output is low, the extrusion of ten or less ova with a definite reddening of the cloaca in one or more of the toads presents very considerable difficulties of interpretation. It is with this type of doubtful positive that the inclusion of the Friedman test proves most helpful.

DISCUSSION.

The advantages of the new extraction method can now be stated in more precise terms. In the first place, the reaction of the urine is adjusted to pH 4·0, maximum adsorption being obtained at the iso-electric point of the gonadotropic hormone. It utilizes, moreover, a specific characteristic in that after centrifugalization, the hormone can be extracted with N/10 sodium hydroxide, while toxic substances from human metabolism, together with bacterial bodies resulting from urines that have undergone septic decomposition, are retained in the kaolin precipitate.

On the other hand, the alcohol or acetone precipitation methods of Zondek (1930) tend to concentrate the toxic and bacterial bodies along with the gonadotropic hormone on the alcoholic precipitate, the aqueous extract of which when injected into the test animal will operate against the normal physiological mechanism, the endocrine system being consequently desensitized by the high degree of toxicity.

Such a state of intoxication leads either to the death of the animal, or if it survives the test, the normal functional activities of the ovaries become so depressed that they are incapable of reacting with the minute quantities of gonadotropic hormone associated with early pregnancy, and the percentage error of false negative results is therefore increased. Further, there is evidence to show that some of the hormone becomes denatured in the course of the acetone or alcoholic treatment.

The method presented here overcomes these difficulties by employing throughout the entire procedure only dilute hydrochloric acid and sodium hydroxide, thus ensuring complete stability of the gonadotropic hormone by choice of selected proportions and sequence of reagents. At the same time a useful by-product of sodium chloride is provided by the interaction of the hydrochloric acid and sodium hydroxide, the presence of which, together with added glucose (and a trace of absolute alcohol for direct intravenous injection with the Friedman test), produces a stimulating effect on the metabolism of the test animal and overcomes the initial shock caused by the injection. It is also important from the point of view of providing for the physiological requirements of the animal in maintaining a high state of general health throughout the time of the test. By this means the endocrine system is undisturbed, the ovaries remaining in a sensitive and freely reactive condition.

Altogether 1000 urines have been tested by this method, and in no case was an incorrect result obtained from the application of the combined Friedman and Hogben tests, so that its accuracy can be said to be a great improvement on native urine injections or with the existing methods of extraction. Two cases of doubtful positivity, however, were recorded by the Hogben test, 7 and 16 ova being expelled in the two cases respectively, while the cloaca of all six toads showed a strong red coloration. Both these cases gave clear-cut positive reactions by the Friedman test. No deaths from toxaemia were recorded throughout the series of tests.

SUMMARY.

A rapid and easy method is decribed for the routine preparation of gonadotropic hormone extracts from human urine for application to the biological pregnancy tests, based on the adsorption properties of kaolin at pH 4·0.

Attention has been given to the physiological requirements of the test animal during the time of the test, and special means have been devised to ensure that the extract does not cause the disturbing symptoms that sometimes arise from native urine injections or extracts prepared by the alcohol or acetone technique.

Urines from 1000 cases in which pregnancy diagnosis was required have been examined by the new process using the Friedman and Hogben tests simultaneously, the results of all cases being in complete agreement, and confirmed by the subsequent clinical condition of the patient.

I have to express my warmest thanks to Dr. J. Patterson for his generous offer in placing at my disposal his stock of South African toads, and for his help and encouragement during the course of this study. My thanks are also

due to Dr. T. W. Stallybrass, County Medical Officer of Health for Dorset, and Dr. T. V. Cooper, Pathologist to the County Laboratory, Dorchester, who kindly allowed the investigation to be continued at the County Laboratory.

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IDENTITY OF HYALURONIDASE AND SPREADING FACTOR.

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Received for publication September 29, 1940.

IN a preliminary communication (Chain and Duthie, 1939) we have presented suggestive evidence for the identity of the so-called spreading factor of Duran-Reynals (1928, 1929) and McClean (1930, 1931) with an enzyme acting on a mucin-like substance present in synovial fluid and vitreous humour. Since the appearance of our preliminary report we have been engaged in collecting more conclusive evidence for the identity of the two substances which we present in the following paper. The evidence is based on the facts that (1) the enzyme has been demonstrated to be present in all sources of spreading factor investigated, and an immediate spread in the skin has never been observed in solutions not containing the enzyme; (2) there exists a satisfactory quantitative correlation between enzyme activity and spreading activity; (3) a mucin-like substance has been isolated from skin with properties similar to those of vitreous humour and synovial fluid, and is acted upon by solutions containing spreading factor in the same way as the substances in vitreous humour and synovial fluid.

While this work was in progress a number of publications arising from our preliminary report have appeared, partly confirming our results and partly anticipating some of the work on which we were actually engaged (Favilli, 1940; McClean and Hale, 1940; Meyer *et al.*, 1940*b*, *c*; Claude, 1940; Madinaveitia, 1940; Madinaveitia *et al.*, 1940). With the exception of the last-mentioned all the investigators agree with our view of the existence of a close relationship between spreading and enzyme activity.

Meyer and Palmer (1936) have isolated from vitreous humour and umbilical cord a protein-free polysaccharide composed of glucuronic acid and N-acetylglucosamine in equimolecular proportions. This substance was termed hyaluronic acid. In a later communication Meyer, Smyth and Dawson (1939) were able to show that a substance with identical chemical properties, but

with a much higher viscosity, was present in synovial fluid and was, in fact, responsible for the two main characteristic properties of this fluid, its high viscosity and string formation after the addition of acetic acid.

We have prepared purified hyaluronic acid according to the prescriptions of Meyer, and his collaborators, both from vitreous humour and synovial fluid. We have found that the prepared substances were attacked by the "mucolytic" enzyme present in spreading factor solutions of different origin and, in fact, were the only substances in vitreous humour and synovial fluid acted upon by the enzyme. It is therefore certain that hyaluronic acid is the substrate for the "mucolytic" enzyme in spreading factor solution which accordingly is termed "hyaluronidase."

The purified polysaccharide acid from synovial fluid gave very viscous solutions, and the effect of the mucolytic enzyme on it was the same as on the original unpurified synovial fluid, consisting of an immediate fall of viscosity, followed later by a liberation of glucuronic acid and N-acetylglucosamine. The purified polysaccharide acid from vitreous humour gave much less viscous solutions than the polysaccharide acid from synovial fluid. Meyer (1938*a*) has suggested that the differences in viscosity between the polysaccharide acids may be due to differences in their degree of polymerization, the vitreous humour hyaluronic acid being partially depolymerized by a hyaluronidase present in the ciliary body. Though we have not been able to confirm the presence of the enzyme in the ciliary body of ox and sheep eyes, it is possible that the depolymerization of vitreous humour by hyaluronic acid may have been caused by some other, perhaps non-enzymatic substance (see Robertson *et al.*, 1939). The depolymerization of hyaluronic acid, as it occurs in vitreous humour, is, however, only partial, and it is acted upon, like synovial fluid hyaluronic acid, by all spreading factor containing solutions, with complete depolymerization, as demonstrated by the rapid and complete loss of its original moderate viscosity, followed by the liberation of glucuronic acid and N-acetylglucosamine.

METHODS.

Tests for Hyaluronidase Activity.

Hyaluronidase activities can be studied by three methods :

(1) By measuring the rate of decrease in viscosity of hyaluronic acid solutions when acted upon by the enzyme.

(2) By measuring the increase in reducing substances produced from hyaluronic acid by the action of the enzyme. This method can be used as a specific test only with protein-free hyaluronic acid preparations, since proteolysis leads to the liberation of reducing substances (Hewitt, 1938).

(3) By measuring the increase in N-acetylglucosamine from hyaluronic acid by the action of the enzyme.

The rate of liberation of reducing substances and N-acetylglucosamine is too small to be measured accurately ; hence methods (2) and (3) are not suitable for accurate quantitative estimations of the enzyme. It was found, however, that the rate of decrease of viscosity of hyaluronic acid solutions

from synovial fluid when acted upon by the enzyme under certain conditions was proportional to the concentration of the enzyme within a certain concentration range ; and it was sufficiently rapid and reproducible for a quantitative assay of the enzyme.

Viscometric Determination of the Enzyme.

Enzyme activity was measured with an error of about $\pm$ 10 per cent. in Ostwald viscometers using synovial fluid from the tibio-astragaloid joint of cattle as substrate. The substrate used was prepared in two ways. In some cases freshly aspirated fluid was preserved by the addition of acriflavine to a concentration of 1 : 10,000, and after filtering through pleated filter paper, was adjusted before use to a viscosity 3 to 3·5 times that of water, and to a slightly acid pH by the addition of KH_2PO_4 and saline. It was then used directly, or was stored frozen. If unfrozen it underwent a rapid loss in viscosity after some days, presumably because of infection. In most cases the substrate used was a dried product prepared as follows : The fresh synovial fluid was shaken with chloroform and n-butyl alcohol (Seastone, 1939) and precipitated with two volumes of alcohol. The powder was dried with alcohol and ether, and was kept in a desiccator in the cold. This is essential, since the powder is hygroscopic and therefore becomes easily infected. Yield 2 g. per 100 c.c. 1 g. of the powder was dissolved by grinding and subsequent shaking at 37° C. for a few minutes in 2 c.c. $N/2$ NaOH, and about 70 c.c. of distilled water. The solution was made approximately neutral with $M/5$ KH_2PO_4 and acriflavine to 1/10,000 was added, after which it was filtered through pleated filter paper in the cold. A sample sufficient for one to two days was adjusted to a viscosity about 3·5 times that of water, and to a final molarity of about $M/15$ salt by the addition of freshly filtered phosphate buffer pH 7·0, the remainder being stored at 4° C. as a stock solution. This stock solution undergoes a slow loss in viscosity at 4° C., a solution of a viscosity three times that of water losing some 10 per cent. in 24 hours at 4° C. In carrying out tests 1·5 c.c. of the diluted substrate was measured into four or five test tubes 5·5 cm. by 1·0 cm., using a 1 c.c. record tuberculin syringe and a small hypodermic needle, the tubes being then placed to stand for some time in the bath at 26° C., containing the viscometers. 0·4 c.c. of enzyme in a concentration as judged by preliminary tests sufficient to cause a fall in viscosity between 10 per cent. and 35 per cent. in 30 minutes (1 to 4 units per c.c.) was added, two tubes receiving a standard preparation of known potency. The tubes were corked and rotated vertically on a turntable for five minutes, 1·7 c.c. of the contents being then transferred to the viscometers by a syringe. These were of the Ostwald type, having a bulb capacity of about 0·8 c.c. and capillary 7·5 cm. and 0·05 mm. internal bore. They were made from the same piece of capillary tube, and gave almost identical viscosity values over the range used. As many readings as were possible were taken over a 40 to 60-minute period, and were plotted as multiples of the water time for the viscometer against time from the beginning of mixing. Under these conditions it was found (Fig. 1) that the time required to reach any definite multiple of the

water time was inversely proportional to the concentration of enzyme present, and comparison with the standard preparation was therefore possible.

Several points should be observed. Specimens of substrate which undergo a rapid loss in viscosity (more than 1 second in 5 minutes) should be rejected. As recommended by Madinaveitia (1940), it is better to blow the liquid into the bulb of the viscometer than to suck. Dust particles must be eliminated from all solutions used, by repeated filtering and by taking care during the manipulations, and bubbles should be excluded from the viscometers. Water times should be determined daily, using a water volume equivalent to that of the enzyme-substrate mixture measured. The viscometers should not be heated above the temperature of the bath.

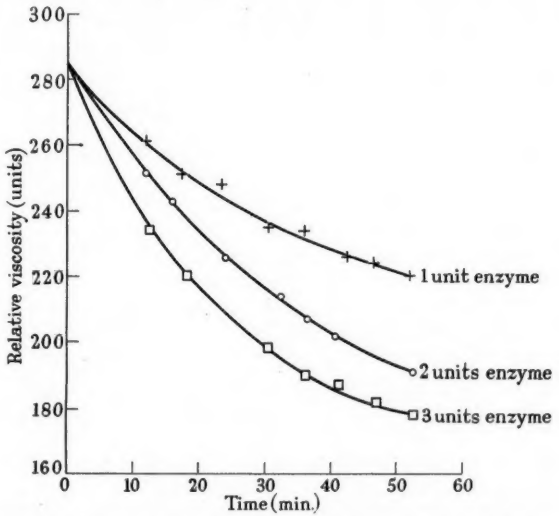

FIG. 1.—Rates of decrease of viscosity of hyaluronic acid caused by different amounts of hyaluronidase.

Choice of Unit.

In all comparisons the standard enzyme solution used was a sterile (filtered) extract of dried testis powder in $M/15$ acetate buffer pH 4·6 containing 1500 units per c.c. In the rabbit skin 0·1 unit in 0·25 c.c. haemoglobin solution gave an area about 1·25 to 1·5 times that of a control haemoglobin solution. 0·05 unit was barely detectable. Viscometrically it was found convenient to carry out determinations using solutions of between 1 and 2 units per c.c., i.e. a final concentration of between approximately 0·2 and 0·4 units per c.c. in the substrate.

Measurement of Diffusing Activity.

White albino rabbits, selected for thin skin and absence of moulting, were clipped on both sides of the vertebral column. Solutions injected were as

follows: 0·25 unit and 0·1 unit of standard testis preparation, 0·1 unit of two or three unknown solutions (first estimated viscometrically) and a control of haemoglobin alone, all in 0·25 c.c. of homologous haemoglobin. Each solution was injected in duplicate with a No. 20 needle. Twenty minutes after each injection the long and short diameters were measured, holding the rabbit skin gently stretched, and the results expressed as the ratio of the products of the diameters to those of the control injections. Similar measurements after 12 and 24 hours were not substantially different, and are not recorded.

Reference to Table I shows that in a series of nine animals there was in every case a clearly recognizable difference between the area of spread produced by 0·25 and 0·1 units of the standard testis preparation.

TABLE I.—*Comparison of Area of Spread Produced by* 0·25 *and* 0·1 *Units of Standard Testis Preparation.*

Animal number.	Spread with 0·25 unit.	Spread with 0·1 unit.	Spread given by 0·25 unit. / Spread given by 0·1 unit.
1	1·30	1·20	1·08
2	1·57	1·37	1·12
3	1·60	1·39	1·15
4	1·65	1·39	1·18
5	1·69	1·23	1·38
6	1·74	1·26	1·38
7	1·82	1·55	1·17
8	1·93	1·49	1·30
9	2·11	1·78	1·18

The figures are multiples of control areas (control area = 1·0), each value representing the mean of two determinations, measured twenty minutes after injection.

Determination of N-acetylglucosamine.

Hyaluronidase action can be detected by the colorimetric determination of N-acetylglucosamine, liberated from hyaluronic acid by the action of the enzyme. Crude hyaluronic acid preparations, still containing proteins, can be used as substrates; in this work hyaluronic acid preparations from vitreous humour, prepared according to the method of Seastone (1939) have been used. Usually, substrate solutions, containing 1–2 mg. of crude hyaluronic acid per c.c., were incubated for 16 hours with the enzyme at pH5 at 37° C., and the liberated N-acetylglucosamine was then determined in 1 c.c. samples. Controls with boiled enzyme were set up as a routine, but invariably negative results were obtained. The determinations were done according to the method of Morgan and Elson (1934). The colour intensities were measured by a Spekker photo-electric cell which was calibrated with solutions of N-acetylglucosamine of known strength.

Material Investigated for the Presence of Hyaluronidase and Spreading Activity.

Organ extracts were prepared by grinding fresh tissues in the homogenizer of Elvehjem and Potter (1936) or in a pestle with sand with one, two or three volumes of *M*/10 acetate buffer pH 4·6. They were subsequently centrifuged and the supernatant filtered and tested. The leeches used were chopped into pieces and ground in a pestle and mortar with sand, buffer being added. Venoms used were dried products. The specimen of bee sting was obtained through the courtesy of Messrs. Antibody Products Ltd., and represented twelve bee stings per c.c. The organisms tested were 24 to 48-hour cultures grown aerobically or anaerobically in broth, centrifuged at 7000 r.p.m., and preserved in the cold by the addition of acriflavine to 1/10,000. The specimens of *Cl. welchii* toxin tested were obtained from the Wellcome Physiological Laboratories through the kindness of Dr. J. W. Trevan.

Comparison of Viscometric Determinations and Diffusing Activity.

In order to establish the identity of hyaluronidase and diffusing factor we have examined extracts from as many as possible of those sources in which the presence of diffusing factor has been reported by various workers. In all cases qualitative comparisons were made between the amount of enzyme as detected by the viscometric method and diffusing effect. In a number of representative cases summarized in Table II, exact quantitative comparisons were carried out between the spread given in 20 minutes by 0·1 unit of extract determined viscometrically and that given by 0·1 unit and 0·25 unit of the

TABLE II.—*Comparison of Viscometric Measurements with Diffusing Activity of Hyaluronidase Preparations from Various Sources.*

Source.	Enzyme content (units/mg.).	Area of spread of— Solutions containing 0·1 unit hyaluronidase.	0·1 unit standard testis extract.	0·25 standard testis extract.
Dialysed testis extract	245	1·63	1·56	2·12
Dialysed testis extract heated 60 min. at 54° C.	60	1·39	1·26	1·74
Testis extract after $(NH_4)_2SO_4$ precipitation and dialysis	415	1·26	1·23	1·69
Leech extract dialysed	200	1·52	1·56	2·12
Copperhead venom	27 (units/c.c.).	1·32	1·23	1·69
Black tiger venom	111	1·54	1·23	1·69
Bee sting	24	1·26	1·26	1·74
Cl. welchii toxin	220	1·76	1·49	1·93
Pneumococcus culture	150	1·70	1·49	1·93

The figures are multiples of control areas (control area = 1), each value representing the mean of two determinations.

standard testis preparation. In addition to the viscometric measurements, in the majority of cases determinations of N-acetylglucosamine were carried out.

DISTRIBUTION OF HYALURONIDASE AND SPREADING FACTOR ACTIVITY.

Tissue Extracts.

Testis was the only mammalian organ showing either hyaluronidase activity or immediate spreading in the dermis. In the cases of certain organ extracts such as sheep spleen, ox ciliary body and rabbit pancreas, slight activity was sometimes observed both in the skin and viscometrically. Repeated experiments using freshly prepared tissues were entirely negative, and we are obliged to conclude that the few positive results were due to bacterial contamination. The following were the organs examined in the rabbit—lung, liver, kidney, brain, spleen, pancreas, muscle and preparations of polymorphonuclear leucocytes and lymphocytes. In the ox we examined ciliary body, pituitary lymph glands and thymus as well as those organs just listed for the rabbit. In the sheep we examined spleen and ciliary body. Tissue from the Jensen rat sarcoma was also investigated. As will be seen from Table II purification of testis extract by dialysis and precipitation with $(NH_4)_2SO_4$ as described below, or partial destruction by heat, did not alter the relationship between hyaluronidase content and diffusing activity. These results contradict those recently published by Madinaveitia *et al.* (1940). In their short preliminary note insufficient experimental details are given to enable us to give an explanation of the discrepancy. The partial destruction of hyaluronidase activity was achieved by incubating a dialysed testis extract at 54° C. for 60 min.

No liberation of N-acetylglucosamine from hyaluronic acid was found with any tissue extract except testis. (For pH activity of testicular enzyme see Table IV.) In these experiments 2 c.c. of tissue extracts, prepared by grinding the tissue with one volume of water in the homogenizer of Elvehjem and Potter (1936), were incubated at pH 5 or pH 6 with 5 c.c. of hyaluronic acid, containing 2 mg./c.c.

Bacterial Filtrates.

Of the bacteria examined only *Staph. aureus*, pneumococcus, *Cl. welchii* Types A and B and *Cl. septique* contained any appreciable quantity of hyaluronidase or spreading factor, the most potent being given by *Cl. welchii* Type A. In all cases examined the diffusing activity was definitely greater than would be expected from the enzyme content, corresponding approximately to 0·2 unit instead of the 0·1 injected. Slight enzyme activity (less than 0·2 unit per c.c.) was found in 48 hours' broth cultures of anaerobic streptococci, but as would be expected, there was no diffusing effect at that dilution. No enzyme or diffusing activity could be detected in at least 25 strains of *Strept. haemolyticus*, the majority of which were virulent. In several strains of *Strept. viridans*, meningococcus, *Bact. coli* and *Cl. welchii* Type C and D, neither enzyme activity nor diffusing effect was found.*

* Many of these determinations of hyaluronidase and spreading activity in bacterial filtrates were carried out by Dr. J. M. Barnes, to whom we are indebted for placing his results at our disposal.

After this work was completed papers by Robertson, Ropes and Bauer (1940) and Meyer *et al.* (1940*a*) dealing with hyaluronidase activity in bacterial cultures have appeared. Their results agree substantially with ours except for the fact that we have never been able to find any hyaluronidase activity whatever in any of the streptococcal cultures tested. The liberation of N-acetylglucosamine from vitreous humour hyaluronic acid was always found to go parallel with the viscometric determinations. The enzyme solutions were added to 5 c.c. of the substrate solutions (2 mg. hyaluronic acid per c.c.) in amounts of 0·1–2 c.c. depending on the strength of the enzyme.

Leech Extracts and Venoms.

Leech extract contained enormous amounts of hyaluronidase and gave a good correlation with diffusing activity. Equally good agreement between hyaluronidase and diffusing activity was obtained with bee venoms. In the case of snake venoms examined the diffusing activity was somewhat stronger than would be expected from their hyaluronidase content. With both bacterial filtrates and venoms the skin injected was definitely thicker (more oedematous) than that which had received testis preparations, and it is possible that the increased permeability of the vessels in the injected area caused by toxic substances may account for the greater area of spread. Leech extract and snake venoms liberated N-acetylglucosamine from hyaluronic acid. Leech extract was added in amounts of 0·1 c.c. to each 5 c.c. of the substrate solutions (2 mg. hyaluronic acid per c.c.), the snake venoms in amounts of 1–2 mg. to each 5 c.c. of the substrate solutions.

Azoproteins.

Azoproteins have been shown to have a spreading effect on the skin similar to that of the testicular spreading factor (Claude, 1935). The spread caused by azoproteins differs, however, from the spread caused by testis spreading factor in that it is very much slower, and is accompanied by oedema. We have diazotized sulphanilic acid and coupled it with ox serum following the prescription of Claude (1935). One volume of the resulting azoprotein solution was diluted with three volumes of distilled water. 0·4 c.c. of this solution were added to 1·5 c.c. of synovial fluid. The synovial fluid used, when diluted with a corresponding amount of 0·9 per cent. saline, had a viscosity of 3·25 times that of water. The azoprotein solution produced a loss in viscosity of 14 per cent. in 30 minutes. Three units of testicular hyaluronidase produced a loss in viscosity of 18 per cent. over the same period. The azoprotein caused spreading in the skin of rabbit, but quantitative comparisons of its spreading and viscosity decreasing power were not carried out, since the mode of spreading of azoproteins differs from that of hyaluronidase and therefore no strict comparisons are possible.

PROPERTIES OF HYALURONIDASE.

Stability.

Contrary to the statements of Madinaveitia the testicular hyaluronidase is stable over a fairly wide pH range. Sterile solutions containing 15 units

per c.c. either in 0·85 per cent. NaCl containing *M*/50 buffer or in *M*/50 buffer alone were incubated twelve hours at 37° C., and when tested viscometrically showed no loss of activity between pH 4·6 and 9. At pH 4 and 9·6 there was some 30 per cent. loss, and the destruction was much greater on either side of this range (90 per cent. at pH 3). Using unsterilized solutions, results similar to those of Madinaveitia's were obtained, and it is likely that the destruction obtained by him was due to bacterial action.

The enzyme is rapidly destroyed by gentle shaking, being most rapidly destroyed in dilute salt solutions, and especially in distilled water. Stability was greatest when shaken in 0·85 per cent. NaCl buffered to 4·6 with *M*/50 acetate as shown in Table III. For these reasons all solutions tested by us were shaken as little as possible and were stored in the cold.

TABLE III.—*Inactivation of Testicular Hyaluronidase by Shaking.*

Hyaluronidase solution contained 1·5 units per c.c. Temperature 25° C.

Diluting medium.	Percentage activity after 3 hours.	
	Shaken.	Unshaken.
0·15 *M* NaCl + 0·02 *M* acetate buffer pH 4·6 .	50	100
0·15 *M* NaCl + 0·02 *M* phosphate pH 6·0 .	0	100
0·15 *M* NaCl + 0·02 *M* phosphate pH 7·0 .	0	100
0·02 *M* acetate buffer pH 4·6. . . .	0	100

Effect of Antiseptics.

Hyaluronidase is not affected by being left in contact with toluene without shaking, but is markedly inhibited by chloroform under these conditions.

The amount of enzyme destroyed by shaking is greater in the presence of chloroform or toluene, as shown in the following experiment. 2 c.c. of testicular enzyme solution, containing 15 units per c.c., showed no loss over a period of one hour at 37° C. in the presence of chloroform or toluene, if unshaken ; the same solution shaken without either antiseptic showed a 70 per cent. loss of activity ; if toluene or chloroform were added, the loss of activity was approximately 100 per cent. The amount of antiseptic added was 0·1 c.c. to 2 c.c. of enzyme ; the tubes were revolved in a vertical plane at 80 r.p.m. In view of the sensitivity of the enzyme to even mild shaking, care was taken to shake the solutions as little as possible. Merthiolate, phenyl mercuric nitrate and acriflavine do not affect the activity of the enzyme, and these antiseptics were used for the protection of the enzyme solutions from bacteria.

pH Activity and Salt Effect.

Table IV shows the activity of testis and *Cl. welchii* hyaluronidase at different pH values. Vitreous humour hyaluronic acid was used, prepared according to the method of Seastone (1939). The substrate was used in solutions containing 2 mg./c.c. of buffer. To each c.c. of the substrate solutions were added 0·1 mg. (32 units) of purified testis hyaluronidase dissolved in 0·1 c.c. of water, respectively 0·1 c.c. of a filtrate of *Cl. welchii*, containing 220 units per c.c. The buffers were *M*/10 citrate buffers for pH 2–4, *M*/10 acetate buffer for pH 5, *M*/10 phosphate buffer for pH 6 and pH 7, *M*/10 borate buffer for pH 8 and pH 9. The substrate solutions were incubated with

the enzyme for 16 hours at 37° C. and the liberated N-acetylglucosamine was determined colorimetrically.

TABLE IV.—*Effect of pH on Hyaluronidase Activity.*

Hyaluronidase from	γ/c.c. N-acetylglucosamine liberated at pH							
	2.	3.	4.	5.	6.	7.	8.	9.
Testis . . .	0	50	100	270	150	120	65	0
Cl. welchii . .	0	0	265	280	487	532	235	55

Both testicular and bacterial hyaluronidase are active over a fairly wide range of pH. The optima of the two enzymes are slightly different, pH 5 for the testicular and pH 6 for the bacterial enzyme.

The activity of the testicular hyaluronidase at different pH values is influenced by the salt concentration, as shown in Fig. 2. *M*/5, *M*/15 and *M*/90 buffers were used, and the amounts of liberated N-acetylglucosamine were determined after 16 hours' incubation of the substrate with testis hyaluronidase. Concentrations of enzyme and substrate and the types of buffers used were the same as in the experiments recorded in Table IV. The enzyme in diluted buffers is much more active at lower pH and much less active at higher pH than the enzyme in stronger buffers.

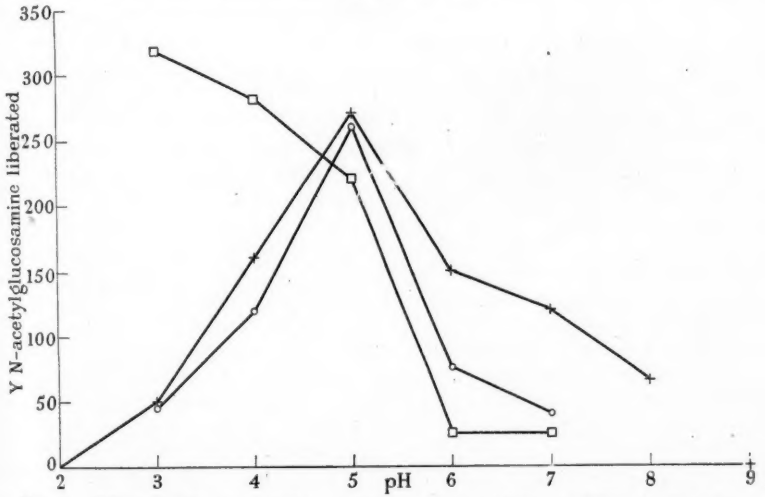

FIG. 2.—Effect of salt concentration on hyaluronidase activity at different pH values: — □ — = *M*/90 buffers. — + — = *M*/15 buffers. — 0 — = *M*/5 buffers.

Effect of Inhibitors.

The possible inhibitory action on hyaluronidase of a number of substances was tested as follows: *M*/100 solutions of sodium fluoride, potassium cyanide, sodium arsenite, iodine and sodium iodoacetate were prepared containing 1·5 units per c.c. of standard testis preparation and added to substrate (0·4 c.c.

enzyme to 15 c.c. substrate) and tested immediately in the viscometer for activity. At this final dilution of $M/500$ inhibitor there was complete inhibition only by iodine, the enzyme activity not being restored by the addition of sodium bisulphite, and a 50 per cent. loss of enzyme activity in the case of iodoacetate. Fluoride, arsenite and cyanide had no effect on enzyme activity. On the other hand both $(NH_4)_2SO_4$ and Na_2SO_4 produced considerable destruction in comparatively short periods. The addition of two volumes of a saturated solution of Na_2SO_4 or one volume of a saturated solution of $(NH_4)_2SO_4$ produced some 50 per cent. loss in 12 hours in strong solutions containing 3000 units per c.c. The activity was not restored on dialysis.

Partial Purification of Enzyme.

Because of the instability of the enzyme in the presence of the usual protein precipitates it has not been possible to carry its purification very far. A preparation having an activity of about 400 units per mg. equal to that prepared by the lead hydroxide method of Morgan and McClean (1932) was prepared as follows :

Dried bull testicle powder (obtained from the Instituto Biologico Argentino, Buenos Aires) was extracted three times, twice with six volumes, and once with three volumes of $M/50$ acetate buffer pH 4·6. The combined extracts (14 per cent. dry weight) had an activity of about 2500 units per c.c. The extract was evaporated to one-fifth of its volume in a large dish by passing a stream of warm air over the surface at 37° C., and was centrifuged. It was then dialysed with the minimum amount of shaking against tap water containing $M/50$ buffer pH 4·6, and, after centrifuging, precipitated with an equal volume of a saturated solution of $(NH_4)_2SO_4$. The precipitate was centrifuged off immediately, and the supernatant dialysed against distilled water in the cold. The solution containing $M/50$ acetate buffer pH 4·6 was dried from the frozen state *in vacuo*.

Isolation of a Substance closely Resembling Hyaluronic Acid from the Skin of Rabbits.

Five rabbit skins were clipped and then depilated with barium sulphide. They were thoroughly washed with running tap-water, minced through an ordinary mincer, treated twice with acetone (24 hours each time) and once with ether, dried first on filter paper and finally over P_2O_5. Portions of 100 g. of the dried skin powder were extracted twice with $N/100$ NaOH (600 c.c. and 750 c.c.) at room temperature for eight hours. The extracts were brought to pH 7·4, concentrated to 65 c.c. and precipitated with 600 c.c. of acetone. The precipitate, after drying in a desiccator, was taken up in portions of 2·7 g. in 20 c.c. of water and shaken for several hours. The insoluble residue was centrifuged and extracted with 10 c.c. water. The very viscous solution was dialysed against distilled water for 27 hours. The dialysed solution was concentrated to 30 c.c. and precipitated with 300 c.c. of acetone. The precipitate, after washing with acetone and ether, was dried over P_2O_5. The material dissolved in water to a 2 per cent. solution with a viscosity of 4 (water = 1). The viscosity was decreased to that of water by the addition of 320 units of testis hyaluronidase solution to 5 c.c. of a 2 per cent. solution

of the skin substance in phosphate buffer pH 6 within 30 minutes at room temperature.

After incubation at 37° for 16 hours 150 γ/c.c. of N-acetylglucosamine were set free.

Effect of Testis Hyaluronidase on Sexual Skin Mucin.

Ogston *et al.* (1940) have investigated some properties of a mucin-like substance accumulating in the sexual organs of female monkeys during the oestrous phase (Aykroyd and Zuckermann, 1938). This mucin resembled the mucin from synovial fluid, giving extremely viscous, string-forming solutions, precipitable by acetic acid in the same typical way as synovial mucin. We therefore decided to test the effect of testis hyaluronidase on a sample of this mucin, kindly given to us by Dr. Zuckermann. The result of the addition of testis hyaluronidase was an immediate fall in viscosity to that of water, loss of precipitability with acetic acid, liberation of reducing substances, consisting of N-acetylglucosamine and glucuronic acid. The mucin of sexual skin must therefore be hyaluronic acid or a substance closely related to it.

Specificity of Testis Hyaluronidase.

As was to be expected the testis hyaluronidase is strictly specific, acting only on hyaluronic acid. Other mucins, e.g. salivary mucin, gastric mucin, duodenal mucin, the substrate for lysozyme, are not acted upon. Salivary mucin was prepared from cow salivary gland by extraction with water; gastric and duodenal mucins were obtained from fistulae of the cat and pig (kindly given to us by Mrs. M. A. Jennings and Prof. H. W. Florey); the substrate for lysozyme was prepared by Mr. L. A. Epstein, in this Department, from *M. lysodeikticus* (Epstein and Chain, 1940).

Kurzrok and Miller (1928) made the observation that semen contained an enzyme capable of dissolving the cervical plug mucin and thus enabling the spermatozoa to penetrate into the uterus. We have tested whether hyaluronidase was identical with this enzyme. 0·2 c.c. of mucin obtained from the uterine cervix of a cow (given to us through the kindness of Dr. Walton of the Institute of Animal Pathology, Cambridge) were incubated with 1 c.c. testicular hyaluronidase (containing 4000 units) at 37° C. for one hour without any effect. Similar results were obtained with specimens of human cervical plug, very kindly collected for us by Prof. J. C. Moir, of the Nuffield Department of Gynaecology and Obstetrics. Kurzrok and Miller themselves have tested extracts from bulls' testicles for the presence of the enzyme, with negative results.

DISCUSSION.

In extensive comparisons between the hyaluronidase activity (determined viscometrically) and spreading activity in the skin of a large number of animal organs, numerous bacterial filtrates, snake venoms, leech extracts and azoproteins, we have noticed in all cases a strict qualitative correlation between the two. There was always spreading in the skin when hyaluronidase was present, and in no case was spreading activity noticed in the absence of hyaluronidase activity.

Quantitatively good agreement was found when spreading activity and hyaluronidase activity of testis extracts of different enzyme strength, leech extracts and bee sting venom were compared. In the cases of some of the bacterial filtrates, especially with *Cl. welchii* and the pneumococcus, and some snake venoms a somewhat bigger spread in the skin was observed than would have been expected from the hyaluronidase activity of the preparations, solutions containing 0·1 unit of hyaluronidase activity giving rise to a spread corresponding to a figure between 0·1 and 0·25 unit when injected into the skin. This moderate inconsistency is easily accounted for by the fact that injection of snake venoms and bacterial filtrates containing toxins was always followed by considerable oedema, due to capillary damage. We have observed that many substances, such as proteolytic enzymes, nucleic acid, arsenious acid, histamine and the permeability increasing polypeptide (Duthie and Chain, 1939), when injected under the skin, produce oedema and some spread, which, however, appears much later than the typical spread caused by testis extracts or bacterial filtrates, and does not show the immediate flattening of the injected bleb, characteristic of solutions containing spreading factor. This non-specific spread may have been confused in the past with the true spreading effect, giving rise to false positive results. This must have been the case with animal organs and Jensen rat sarcoma tissue, which in our experience were found to be entirely devoid of both hyaluronidase and spreading activity.

The supposition that hyaluronidase and spreading factor are identical postulates the existence of a substance, closely related to synovial hyaluronic acid, in the skin of rabbits. The assumption of the existence of such a substance was proved correct by the isolation of a highly viscous substance from the skin of rabbits which is acted upon by hyaluronidase in the same way as hyaluronic acid from vitreous humour and synovial fluid, with an immediate loss of its viscosity and the liberation of N-acetylglucosamine.

A bleb of fluid injected into the skin retains its shape because it is walled off by jelly-like connective-tissue substance, and is submitted to the tensile force of the stretched fibres (Bensley, 1934). We consider that the removal of a highly viscous compound from this tissue must necessarily result in the passage of fluid through the wall of the bleb, bringing about its flattening and the spread of contained fluid through the adjacent tissues. These are two typical manifestations of spreading factor, and we conclude therefore that spreading factor and hyaluronidase are identical.

However, since the spread in the skin caused by hyaluronidase can be influenced by non-specific irritants, it cannot possibly be represented correctly by the viscometric determinations of hyaluronidase alone.

For the understanding of the physiological function of hyaluronidase it seems to us to be important and significant that the enzyme occurs exclusively in testis. It is not identical with the mucolytic enzyme in semen described by Kurzrok and Miller, which dissolves the mucinous plug in the uterine cervix and thus facilitates the penetration of the spermatozoa into the uterus. No trace of hyaluronidase has been found in any other mammalian organ when prepared freshly, and it seems probable that the contrary findings of Meyer *et al.* (1938*b*, 1940*a*), who found hyaluronidase activity in ciliary body and spleen, were due to bacterial contamination.

We would point out that hyaluronidase cannot be regarded as one of the causes either for the rapid growth or for the invasiveness of tumours since the Jensen rat sarcoma, like all other mammalian tissues, is devoid of it.

Skin hyaluronic acid has an important function with regard to permeability of the skin. It is therefore possible that certain states of increased skin permeability, occurring in infectious diseases such as can be demonstrated by the test of Aldrich and McClure (1924), may be due to a decrease of the hyaluronic acid content of the skin. This may be caused either by the action of bacterial hyaluronidases or by a decreased hyaluronic acid synthesis. It seems that the amount of hyaluronic acid present in the skin is subject to the influence of hormones. This is especially pronounced in the sexual skin of monkeys during the oestrous period. In this connection Sprunt *et al.* (1938*a*, *b*) found a decrease of the spread of ink in the skin of rabbits injected with oestrogenic hormone. The thyroid gland may also be of importance for the regulation of the hyaluronic acid content of skin since there seems to be an accumulation of hyaluronic acid in the skin in myxoedema.

SUMMARY.

1. Evidence for the identity of the spreading factor of Duran-Reynals and McClean with the enzyme hyaluronidase is presented. This enzyme acts on the polysaccharide acid hyaluronic acid present in synovial fluid and vitreous humour and responsible for the mucinous character of these liquids. The enzyme hydrolyses hyaluronic acid with the liberation of N-acetylglucosamine and glucuronic acid. The high viscosity of hyaluronic acid is quickly reduced to that of water.

2. A viscometric method for the determination of the enzyme has been worked out. It is accurate to $\pm$ 10 per cent.

3. Hyaluronidase has been found in all sources of spreading factor. No spreading activity was encountered in the absence of hyaluronidase. Testis was the only mammalian organ in which the enzyme occurred.

4. In quantitative comparisons between hyaluronidase activity determined viscometrically and spreading activity in the skin of rabbits, good agreement was obtained with testis hyaluronidase of different strengths, and with leech and bee venom hyaluronidase. With bacterial and snake venom hyaluronidase somewhat bigger spreads were obtained than were expected from their hyaluronidase content. The reason for this is most probably the oedema caused by these fluids.

5. A substance closely resembling hyaluronic acid was isolated from the skin of rabbits. It was acted upon by testis hyaluronidase in the same way as is hyaluronic acid.

6. The mucinous substance which accumulates in the sexual skin of monkeys during the oestrous phase is acted upon by hyaluronidase and is therefore closely related to or identical with hyaluronic acid. Salivary, gastric and duodenal mucin and mucin of the uterine cervix are not attacked by hyaluronidase.

7. Stability and pH activity of hyaluronidase have been investigated.

The work has been made possible by a grant from the Rockefeller Foundation to this Department for expenses and provision of technical assistance, and by a grant to one of us (E.S.D.) from the Irish Medical Research Council for a technical assistant during part of the time. During the latter part of the work E.C. held a personal grant from the Medical Research Council. We have been greatly assisted by the continued kindness of Dr. D. McClean, Lister Institute, who provided us with material and showed a keen interest in the development of the work. We are also indebted to the Oxford and District Co-operative Society for facilities in the collection of material from their abattoir, to Dr. S. Zuckerman for the specimen of sexual skin mucin, to Dr. A. H. Cruikshank and Dr. R. L. Vollum for bacterial cultures, and to Dr. J. W. Trevan of the Wellcome Physiological Laboratories for the specimens of *Cl. welchii* toxin examined. We wish to express our thanks to Miss F. Gochsheimer and Mr. D. S. Callow for valuable technical assistance.

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SOME OBSERVATIONS ON THE PREPARATION AND PROPERTIES OF THE SUBSTRATE OF LYSOZYME.

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Received for publication October 28, 1940.

In 1922 Fleming discovered a substance in egg white, minute amounts of which were capable of dissolving certain bacteria. This substance, lysozyme, is widely distributed in numerous animal organs and secretions and in plants. In view of its theoretical interest in connection with the problem of immunity the substance has attracted a good deal of attention, and a number of publications dealing mainly with its bacteriological and clinical aspects have appeared. Two reviews on the literature on lysozyme have been published (Mesrobeanu and Naeppel, 1938 ; Yermoljeva and Bujanskaja, 1938).

After a number of conflicting statements in the literature on the chemical nature of lysozyme it was conclusively established by Meyer, Thompson, Palmer and Khorazo (1936) and at the same time independently by Roberts (1937) that lysozyme was a protein. Roberts obtained a purified preparation of higher activity than Meyer *et al.* Abraham and Robinson (1937) starting with Roberts' material obtained crystals from it which did not differ appreciably in activity from the original amorphous preparation. Some of the chemical and physico-chemical properties of lysozyme were studied by Abraham (1939). In view of the protein nature of lysozyme and its activity in extremely high dilutions it became obvious that lysozyme was an enzyme, and the question of the chemical nature of its substrate arose. Preliminary work on this problem was carried out in 1934–35 by Harrison in the Department of Pathology, Sheffield University, and by Roberts in this Department. Harrison noticed a rise in reducing power after the action of a crude preparation of lysozyme on boiled *M. lysodeikticus*, which, in contradistinction to the unboiled organism, is dissolved only to a very slight extent. By dissolving *M. lysodeikticus* in hypochlorite solution and subsequent alcohol precipitation, Harrison obtained fractions whose reducing power was increased by incubation with lysozyme. We were able to confirm Harrison's results using Roberts' purified lysozyme preparation, and proceeded to work out a method of obtaining the substrate of lysozyme in larger amounts in order to examine its properties more closely.

While this work was in progress a paper by Meyer, Palmer, Thompson and Khorazo (1936) on the mechanism of lysozyme action appeared. By mild alkaline hydrolysis these authors obtained fractions from *Sarcinae* which were

acted upon by a lysozyme preparation with the liberation of reducing substances. The fractions contained nitrogen, but were free from proteins and were considered to be nitrogen-containing polysaccharides of the mucoid class (for nomenclature see Meyer, 1938); no details of their chemical properties were given. Increase of reducing power obtained after incubation of chemically inhomogenous substances (even with purified lysozyme preparations) suggests, but does not prove conclusively, the carbohydrate nature of lysozyme. Further chemical characterizations of the substrate or of the substances produced from it by the actions of lyzosyme seemed to us essential for the purpose of establishing conclusively the nature of lyzosyme action. Work was therefore continued on these lines, but was interrupted by the outbreak of war. As we cannot resume investigations for an indefinite time, it has been decided to publish the present account in spite of its incompleteness in parts.

EXPERIMENTAL.

Large Scale Growth of M. lysodeikticus.

Of the micro-organisms sensitive to lysozyme *M. lysodeikticus* was found to be the best starting material for the isolation on a fairly large scale of the substrate of lysozyme. Even the largest increases in reducing matter (calculated in glucose equivalents) brought about by the action of lysozyme were small, varying from 0·5 to 1 per cent. of the total dry weight of bacteria; only a small yield of the actual substrate could therefore be expected. A method had therefore to be developed for obtaining large amounts of *M. lysodeikticus* cells. This organism grows much more easily on a solid than in a liquid medium, and of a number of solid media tried, the following gave by far the best yield of bacteria: Agar, 5 per cent.; glucose, 1 per cent.; autolysed yeast, 20 per cent.; pepsin digested horse meat, 20 per cent.

As far as we are aware only one method for the large-scale growth of bacteria on a solid medium has been described. It is the method of Morgan (1937), and consists of growing the bacteria on agar in large flat metal trays. The trays take up a considerable amount of space, and, in our experience, can get contaminated comparatively easily. The following method (of which the principle was suggested to us by Col. R. F. Bridges) overcomes both these difficulties. It consists in coating the inside surface of ordinary Winchester quart bottles with a thin layer of agar, and seeding bacteria evenly over the surface. In order to make it possible to put up at least 20 bottles at a time, a machine was constructed which rotates the bottles while the agar is cooling, thus distributing it evenly over the inner surface. The machine is also used for seeding the bacteria and for washing off the growth with saline.

A diagram of the machine is given in Fig. 1. It consists essentially of three rows of horizontal revolving racks, each consisting of three brass rods fixed in cast iron end plates, from the outer surface of which project driving shafts. The bearings in which these shafts run are supported in a rigid frame of angle iron. The rods of each rack form a cradle in which may be placed three Winchester quart bottles, so that nine bottles can be revolved at a time. The bottles are held in place by a simple elastic strap. A quarter horse-power

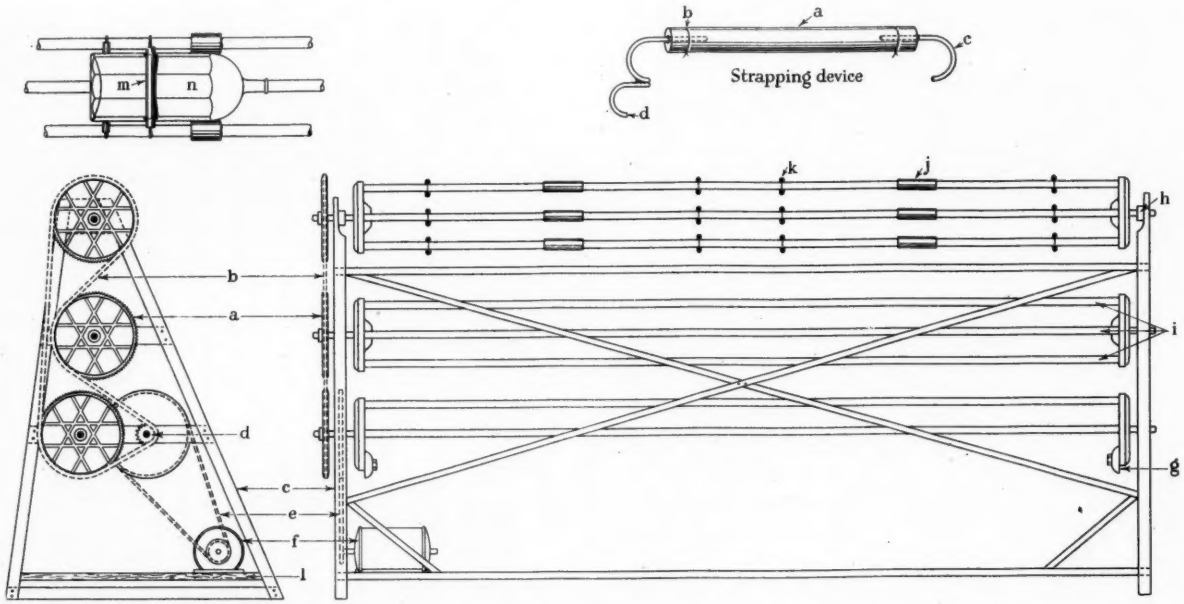

FIG. 1.—Diagram of rotating machine. *a*, bicycle cog wheel; *b*, bicycle chain; *c*, iron frame; *d*, gear wheel (6 : 1); *e*, belt to motor; *f*, ¼ H.P. motor; *g*, lead balancing weight; *h*, brass bearing; *i*, brass rods; *j*, rubber hose buffer; *k*, pressure tubing, bound on with wire; *l*, wooden floor board; *m*, strapping device; *n*, Winchester bottle.

Strapping device: *a*, pressure tubing; *b*, wire; *c*, brass hook; *d*, brass catch.

motor mounted at the base of the frame is geared down to turn the racks, which are coupled together by a bicycle chain, at 15 r.p.m.

Twenty Winchester quart bottles are set up at a time. About 200 c.c. of warm medium of the above composition is poured into each bottle, which is then sterilized by autoclaving. The medium is allowed to cool down to approximately 50° C., and the bottles are put into the machine and rotated until the agar has set. The surface of the agar is dried off by leaving the bottles in the incubator overnight. A thick saline suspension of a 24-hour growth of *M. lysodeikticus* is then introduced into the bottles and they are again rotated in the machine to seed the whole surface evenly. The bottles are incubated at 37° C. for four days, after which the growth is washed off the surface of the agar with saline by rotating the bottles in the machine; the suspension is poured through glass wool to remove larger pieces of agar, is centrifuged at 2000 r.p.m., washed twice with saline, once with acetone and dried in a stream of warm air in a Faust-Heim drying apparatus in large Petri dishes. If a thin layer is taken the material can be scraped off easily; it is powdered in a ball mill in batches of 100 g. and stored. For the purpose of preparing the substrate for lysozyme it can be kept for at least three years. The yield is about 1·5 g. per bottle, i.e. 30 g. per 20-bottle batch. It is therefore not difficult to obtain 100 g. of dried bacteria per week. This method has been successfully used for the large-scale growth of a considerable number of bacteria, such as staphylococci, meningococci, *Ps. pyocyanea*, *B. anthracis*, *Br. abortus*, etc. The yields vary, of course, with the organism.

Tests for the Presence of the Substrate of Lysozyme.

(1) *Estimation of reducing power.*

At the beginning of this work the only known property of the substrate of lysozyme was the appearance of reducing matter after its incubation with lysozyme. This property was used for the detection of the substrate. The tests were usually carried out by mixing 10 c.c. of a 1 per cent. solution in phosphate buffer pH 6·8 of the fractions to be tested with 1 c.c. of a 3 per cent. solution of lysozyme in water (a drop of acetic acid being added to facilitate the solution of the enzyme) and incubating overnight. Reducing power was determined in 1–2 c.c. samples by the ceric sulphate method of Miller and van Slyke (1936) at the beginning and end of the incubation period, and control determinations of reducing power with heat-inactivated lysozyme were always carried out as a routine. With the less pure preparations deproteination was at first carried out, but it was found during the course of the work that all protein precipitants used carried down a certain, sometimes considerable, amount of the reducing substance liberated by the action of lysozyme. Deproteination was therefore entirely omitted. This could be done without any difficulty with the purer preparations, and consistent results were obtained in spite of relatively high blank values.

(2) *Determination on N-acetylhexosamine.*

During the course of the work it was found that the substrate for lysozyme contained a N-acetylated amino sugar which was liberated after incubation

with lysozyme. This substance can be estimated by the method of Morgan and Elson (1934) for the determination of N-acetylglucosamine, which thus presents a simple and quick means for the detection of the substrate of lysozyme. The quantitative figures for the determinations were not always absolutely reliable because of the presence in crude preparations of substances retarding the colour formation.

Lysozyme.

The lysozyme used in this work, which was prepared and purified by the method of Roberts (1937) and given to us by Dr. Abraham, possessed an activity of 2000 units/mg.

Preparation of the Substrate of Lysozyme from M. lysodeikticus.

In order to isolate the substrate of lysozyme in a water-soluble form from the lysozyme-sensitive *M. lysodeikticus* the following methods, developed by various workers for the isolation of the specific carbohydrates, were tried :

Mechanical disintegration of the bacteria followed by extraction with organic solvents, or by digestion with proteolytic enzymes ; extraction of the intact bacterial bodies with organic solvents or trichloracetic acid or with acid and alkali ; dissolution of the bacterial bodies in alkaline hypochlorite (antiformin) or in formamide.

Of these methods only the dissolution procedures proved successful.

(1) *Hypochlorite method.*

The solvent action of alkaline hypochlorite on bacteria was first described by Uhlenhuth and Xylander (1908). Various authors (Altmann and Schultz, 1909 ; Krumwiede and Noble, 1918 ; Furth and Landsteiner, 1928, 1929) have used this reagent for preparing antigenic substances from various bacteria.

M. lysodeikticus was easily dissolved by alkaline hypochlorite. As the substrate for lysozyme is sensitive to the action of alkali at higher temperatures, the dissolution of the bacteria was carried out at 0° C. At this temperature no effect of even strong alkali was noted. Crude substrate preparations were obtained from *M. lysodeikticus* by hypochlorite treatment as follows : Batches of 30 g. of dried organism are added with ice-cooling to 900 c.c. of antiformin solution (B.P. Codex, 1934) with vigorous stirring. A strong evolution of gas begins after a short time and continues for about 5 min. Larger particles are broken up with a glass rod and finally a clear yellow solution is obtained. This is neutralized with strong hydrochloric acid, the temperature never being allowed to rise above 5° C. The neutral point is easily recognized by the fact that at pH 6 a white precipitate begins to appear. The neutralized solution is dialysed for two days in the ice chest against running tap water in a rocking dialyser, similar to that described by Kunitz and Simms (1927). The precipitate which appears during dialysis is removed by centrifugation and the supernatant solution is concentrated in a Faust-Heim hot-air drying apparatus at 55° C. to about 1/5 of its volume. Ten volumes of alcohol are then added,

the voluminous precipitate is left overnight in the ice chest and washed once with alcohol. The material prepared in this way still contains a certain amount of hypochlorite which cannot be removed by repeated washing. Since the remaining hypochlorite interferes with the determination of reducing power and with lysozyme activity, it is removed by suspending the precipitate in alcohol, and passing sulphur dioxide through the suspension for a few minutes. The suspension is then washed twice with 90 per cent. alcohol and twice with absolute alcohol and finally dried *in vacuo* over 60 per cent. NaOH (to remove the last traces of SO_2) and P_2O_5. The preparation thus obtained is an almost colourless powder and gives a rise in reducing power of approximately 1·2 per cent. (calculated in glucose equivalents) after incubating with lysozyme at pH 6·8 for 18 hours at 37° C.

(2) *Formamide method.*

Hot formamide was first suggested by Fuller (1938) as a means of dissolving bacteria for the purpose of obtaining specific carbohydrates. He has used this solvent successfully for the preparation of the group antigen of haemolytic streptococci. We found that *M. lysodeikticus* was dissolved easily by hot formamide ; from this solution a crude substrate preparation could be obtained. The method used was as follows : 16 g. of dried *M. lysodeikticus* are ground into a homogeneous suspension with 200 c.c. of formamide and heated to 170° C. After 15 minutes most of the bacteria are dissolved. The solution is cooled, a small insoluble residue is centrifuged off and eight volumes of acetone are added to the supernatant solution, which is placed overnight in the ice chest. A brown gum separates, which after centrifugation and washing with alcohol becomes granular. It is extracted three times with 100 c.c. portions of hot water and the combined extracts are concentrated *in vacuo* to about a third of their volume. Three volumes of alcohol are added and a protein fraction, containing very little substrate, is precipitated. This precipitate is allowed to settle out in the ice chest overnight, centrifuged off and discarded. To the supernatant liquid three volumes of acetone are added. A brown gum, containing most of the substrate, is precipitated. After standing in the ice chest overnight it is centrifuged off, washed with alcohol and dried. The brown powder thus obtained gives a rise in reducing power of about 6 per cent. when incubated with lysozyme. The yield is usually 1·3 g. from 15 g. of bacteria. Though the formamide method yields crude substrate preparations quickly and easily, these preparations were unsuitable starting material for further purification, since they contained a brown decomposition product, formed by the high temperature during the process of dissolution of the bacteria, which could not be removed by any method tried.

Fractionation of the Substrate.

A purification of the crude substrate obtained by the hypochlorite method was achieved by fractional precipitation of a formamide solution of the substrate with acid alcohol and acetone. The fractionation was carried out as follows : 10 g. of crude substrate were ground with 125 c.c. formamide in a

mortar; the suspension was shaken for 30 minutes at 37° C., and then centrifuged at 7000 r.p.m. The residue was extracted with 50 c.c. portions of formamide. An insoluble fraction of 0·5 g. remained, but contained no substrate. The formamide solutions were pooled, and with continuous mechanical stirring one volume of acid alcohol (absolute alcohol containing 5 per cent. 2 *N* HCL) was added slowly from a dropping funnel. After keeping overnight in the ice chest the precipitate was collected by centrifugation, washed once with a mixture of equal volumes of alcohol and formamide, twice with absolute alcohol, and then dried in a desiccator over P_2O_5. Four further fractions were obtained in the same way by adding (1) 2 volumes of alcohol, (2) 3 volumes of alcohol, (3) 6 volumes of acetone containing 5 per cent. 2 *N* HCL, and (4) 9 volumes of acid acetone. Each fraction was always allowed to settle overnight in the ice chest before collecting it by centrifugation. The alcoholic fractions contained mainly proteins and polypeptides, whereas the acetone fractions were composed mainly of carbohydrates, which gave viscous solutions in water. The six-volume acetone fraction was the richest in substrate, while the nine-volume fraction contained about 1/2 of the substrate content of the six-volume fraction. Addition of more than nine volumes of acetone brought down considerable amounts of a precipitate containing no substrate. Typical results of a fractionation are given in Table I.

TABLE I.—*Fractional Precipitation with Alcohol of a Crude Substrate Preparation from Formamide Solution.*

Volumes of precipitant.	Precipitant.	Weight of fraction (g.).	Increases in reducing power after incubation at pH 6·8 with lysozyme at 37° C. for 18 hours.	
			As γ glucose.	As per cent. increase (in weight of the fractions).
Fraction insoluble in formamide		0·50	0	0·0
1	Acid alcohol	1·30	40	0·4
2	,, ,,	2·40	60	0·6
3	,, ,,	1·90	110	1·1
6	,, acetone	0·50	1200	12·0
12	,, ,,	0·80	610	6·1
18	,, ,,	10·05	0	0·0
24	,, ,,	4·16	0	0·0

The acetone fractions containing the largest amount of substrate were refractionated from formamide with acetone. The purest preparation thus obtained yielded about 15 per cent. of reducing matter after incubation with lysozyme.

Properties of the Substrate.

The purest fractions of substrate were light brown powders, easily soluble in water giving viscous solutions, soluble in formamide, cold ethylene glycol and dilute alcohol, but insoluble in absolute alcohol, acetone, ether, chloroform,

m-cresol, dry pyridine and dioxane. They were of high-molecular weight structure, being non-dialysable through cellophane and collodion membranes.

They contained no protein, gave clear solutions in 3 per cent. trichloracetic acid and a strong Molisch reaction. After incubation with lysozyme about 50 per cent. of the material became easily dialysable and soluble in methyl alcohol, so that the substrate preparation must have consisted of at least 50 per cent. pure substrate. The remaining 50 per cent. contained a small amount of non-hydrolysed substrate and consisted in the main of other carbohydrates and polypeptides.

Preparation and Properties of the Products of the Action of Lysozyme on the Substrate.

Since it seemed difficult to obtain the substrate in a sufficient degree of purity to draw valid conclusions as to its chemical constitution from direct analysis, it was decided to study the products resulting from the action of lysozyme on it. They can be easily separated from high-molecular impurities and from non-hydrolysed substrate by dialysis or by extraction of lysozyme split substrate with methyl alcohol in the following ways :

(1) Methyl alcohol is carefully dried by refluxing over calcined calcium oxide for several hours and redistilled. 300 mg. of purified substrate preparation are extracted with it in a Soxhlet apparatus, carefully excluding water. After a few hours only about 1 mg. per hour continues to be extracted. The methyl alcohol-extracted substrate, now amounting to 215 mg., is taken up in 10 c.c. water and incubated at 50° C. for 24 hours with 2 mg. of lysozyme. After drying *in vacuo* the residue is re-extracted with methyl alcohol in the Soxhlet for 30 minutes. A clear, slightly yellow solution results, yielding 106 mg. of product on drying. No further material is obtained on further methyl alcohol extraction.

(2) Purified substrate (250 mg.) is taken up in 10 c.c. of water and dialysed at 2° C. for four days against 100 c.c. portions of distilled water, which are renewed twice per day and evaporated to dryness. After 72 hours no more dialysable material passes through the cellophane membrane. The undialysable portion is made up to 10 c.c. and incubated with lysozyme (2 mg.) for 24 hours at 37° C. The greater part of the lysozyme is removed by heat coagulation (heating the solution at 90° C. for 15 minutes). The solution is then dialysed as before against distilled water for two days and the combined dialysate evaporated to dryness on a water bath. The dialysable material thus obtained constitutes 46 per cent. of the original substance.

The split breakdown products were light yellow, very hygroscopic powders. Our aim was the identification of their main constituents by means of crystalline derivatives, but unfortunately, the war interrupted our work before this could be completed. We have, however, the results of a number of quantitative analyses and qualitative sugar identification tests on the split hydrolysis products which were carried out with the intention of getting some indication of their composition, and so facilitating the preparation of crystalline derivatives. These results are now presented.

Elementary analysis.

The substances contained C : 45·4 per cent. ; H : 6 per cent. ; total N (Dumas) 6·4 per cent.

Amino-N.

Only traces of free amino-groups are present in the products of enzyme action. After acid hydrolysis, however, 6·4 per cent. of free amino groups appear. (Amino-N was determined by the modification of the van Slyke method by Warburg *et al.* (1935) ; acid hydrolysis was carried out by heating the material in 2 *N* HCL for four hours at 100° C. in a sealed tube.) As this value is identical with the value for total N (Dumas), all the nitrogen in the breakdown products must be present in the form of amino-groups which, however, are blocked. The biuret reaction was negative so that the presence of peptide linkages is unlikely.

Acetyl.

After acid hydrolysis with 25 per cent. toluene sulphonic acid at 100° C. for three hours (Kuhn and Roth, 1933) the material yielded 11·4 per cent. of acetyl groups. Saponification with methyl alcoholic soda gave the slightly higher value of 13·4 per cent. ; Meyer and Palmer (1936) record a similar experience with hyaluronic acid hydrolysates.

N-acetylhexosamine.

N-acetylated amino-hexoses react with dimethylaminobenzaldehyde after being heated for a short time in alkali, forming a compound with a characteristic pink colour. On the basis of this reaction Morgan and Elson (1934) have worked out a quantitative method for the determination of amino-hexoses. In view of the presence of amino and acetyl groups in the enzyme products, the negative biuret reaction and the frequent occurrence of N-acetylated hexosamines in biologically important polysaccharides, we carried out a determination of N-acetylated hexosamines by the above method. The typical pink colour was developed, and its intensity corresponded to 10·6 per cent. standard N-acetylglucosamine. When the N-acetylhexosamine content was redetermined after acid hydrolysis (four hours with 2 *N* HCL at 100° C. in a sealed tube) and subsequent reacetylation with acetonyl-acetone according to Palmer *et al.* (1937) about 30 per cent. N-acetylglucosamine was found. This value is almost certainly low, as losses occur during the acid hydrolysis. It must therefore be assumed that over 30 per cent. of the products consist of a N-acetylated hexosamine. If all the acetyl groups estimated belonged to N-acetylated amino-groups of hexosamines, about 58 per cent. of N-acetylated hexosamine would be present. Only a part of this would be free (about 10 per cent. of the material), the other part being present in the bound state, probably as oligo-saccharides.

Reducing power.

The reducing power as determined by the ceric sulphate method of Miller and van Slyke was 13·2 per cent., and rose to 41 per cent. after hydrolysis in

2 *N* HCL for four hours in a sealed tube at 100° C. It was observed that the values for reducing power determined before acid hydrolysis were 25–60 per cent. higher when the material was dissolved in acid and then neutralized than when it was dissolved in water.

Keto-hexose.

A number of colour reactions characteristic of ketohexoses were obtained with the products of lysozyme action (see Table II).

TABLE II.—*Comparison of Colours obtained by the Action of Different Reagents on the Hydrolysis Products and on Ketohexoses (Fructose).*

Reagent.	Colour produced with—		Reference.
	Hydrolysis products.	Ketohexose.	
Resorcinol	Pink with amber tint	Pink	Roe (1934).
Orcinol-HCl	Amber	Amber	Dehn, Jackson and Ballard (1932).
Orcinol-H_2SO_4	Orange, slowly turning green	Orange	Pirie (1936).
Ammonium molybdate	Blue	Blue	Dehn, Jackson and Ballard (1932).
Codein	Brown	Brown	Ditto.
Stannous chlorite	Yellow	Yellow	,,
Sodium carbonate (5 per cent)	,,	,,	,,
Phloroglucinol	Burnt orange turning black after alcohol addition	Burnt orange turning black after alcohol addition	,,

In addition to these the furfural tests of Mullikin (1904) with aniline was positive. Although pentoses, rhamnose and hexuronic acids, as well as keto-hexose, form furfural, the presence of the three former sugars has been excluded by negative phloroglucinol, orcinol-HCL and Bial tests. It seems therefore likely that the furfural produced originated from a keto-hexose.

Roe (1934) adapted his resorcinol test as a quantitative method for the estimation of keto-hexoses. When this method was applied to our material, about 3 per cent. of keto-hexose was found. After acid hydrolysis for one hour in 6 *N* HCL at 100° C. the keto-hexose content increased to 14·2 per cent. On further hydrolysis the keto-hexose content fell rapidly. The maximum value of 14·2 per cent. for the keto-hexose content is certainly low as destruction of keto-hexoses is known to take place during heating in acid solution.

From the data given above the most likely conclusion to be drawn is that the products of the action of lysozyme on its substrate are a mixture of mono- and oligo-saccharides, their main constituents being a N-acetylated hexosamine and a keto-hexose. No evidence for the presence of hexuronic acids, galactose, mannose or pentoses has been obtained. Neither the N-acetylated hexosamine nor the keto-hexose have yet been fully characterized and identified with known sugars.

Properties of the Substrate in Bacteria.

There are marked differences in solubility between the substrate in the intact state in the bacterial bodies and after isolation by the hypochlorite or formamide method. The latter product is easily soluble in water, cold formamide and cold ethylene glycol, whereas no trace of substrate is extractable by any of these solvents from the bacterial bodies. Even when the bacterial bodies are disintegrated mechanically almost completely, the bulk of the substrate remains in the fractions which are completely insoluble in water. This is shown in the following experiment: *M. lysodeikticus*, which is resistant to the usual methods of mechanical disintegration such as grinding with sand, repeated freezing and thawing, etc., could be crushed almost completely in the bacterial grinding machine of Booth and Green (1938), most of the organisms being broken up in four hours. Dried *M. lysodeikticus* (7 g.) was suspended in 30 c.c. of water and was ground in this machine for four hours. The suspension was then centrifuged at 7500 r.p.m. Three zones appeared in the centrifuge tube. The first zone was the supernatant liquid which contained approximately 50 per cent. of the total bacterial weight. Only 500 mg. of this were non-dialysable. The second zone on microscopic examination was seen to consist almost entirely of broken-up bacteria. The dry weight was 700 mg. and no dialysable material was present. The third zone consisted of broken-up bacteria mixed with apparently unchanged bacteria in the approximate ratio 3 : 1 ; dry weight 2·5 g., no dialysable material present. Material from each zone was tested for substrate by estimating the increase of reducing power after incubation with lysozyme. The results are summarized in Table III.

TABLE III.—*Occurrence of Substrate in Fractions of Mechanically Disintegrated Bacteria.*

Fraction incubated with lysozyme at pH 6 for 16 hours at 37° C.

	Increase in reducing power (in glucose equivalents) as per cent. of the weight of the fractions.
Soluble (non-dialysable) fraction . . .	1·2
Bacterial debris	3·5
Residue	3·7

When dried *M. lysodeikticus* is digested with pepsin at pH 2 at 37°, about 30–50 per cent. of the total weight goes gradually into solution. The substrate for lysozyme, however, remains entirely in the insoluble residue. It must therefore be assumed that the substrate of lysozyme occurs in the intact or mechanically disintegrated bacteria in a different state from that in which it is isolated from the chemically disintegrated bacteria. It may be present either in a higher polymerized form or may be bound to an insoluble protein resistant to the action of pepsin.

Occurrence of the Substrate of Lysozyme in Different Bacteria.

A number of bacteria, sensitive and insensitive to the action of lysozyme, were grown in large amounts by the method described above. The sensitive organisms included three lysable strains, namely, a Sarcina, a gram-positive bacillus isolated from the air and *Br. abortus*, and two unlysable bacteria, namely, *B. subtilis* and a Gram-negative bacillus isolated from the air. These latter bacteria were not dissolved by lysozyme, but were rapidly killed by it. This was demonstrated by the complete inhibition of the respiration of bacterial suspensions which occurred instantaneously after the addition of lysozyme, and by the fact that the bacterial suspensions were found to be sterile after incubation for one hour with lysozyme. This observation is important in that it shows that lysozyme-sensitivity of bacteria cannot be established by observation of lysis only. The organisms which were in no detectable way affected by lysozyme (no growth inhibition, effect on respiration or bactericidal action) included *Staph. albus*, *Bact. coli*, *Ps. pyocyanea*. 1–2 g. of each of the above dried organisms were dissolved in formamide and precipitated with alcohol and acetone, as described above for the isolation of the substrate from *M. lysodeikticus*. The acetone fractions were tested for substrate by determining colorimetrically the N-acetylhexosamine content after incubation with lysozyme at 37° C. for 24 hours. An increase of the N-acetylglucosamine content over the controls (set up with heat inactivated lysozyme) was taken as an indication of the presence of the substrate of lysozyme. As all the organisms were grown on agar medium, agar itself was tested for the presence of the substrate for lysozyme by treating it in an exactly analogous manner to the bacteria. The results are given in Table IV.

TABLE IV.—*Presence of Substrate in Different Bacteria.*

Organism.	Effect of lysozyme on the organism.	N-acetylhexosamine liberated from polysaccharide fractions after incubation with lysozyme in per cent. of the weight of the fraction.
B. subtilis	Growth inhibition, no lysis	1·9
Br. abortus	Lysis	0·7
Sarcina	,,	0·9
Air organism No. 14	Growth inhibition, no lysis	0·9
,, ,, No. 36	Lysis	0·9
Staph. albus	No effect	Very small
Bact. coli	,,	,,
Ps. pyocyanea	,,	,,
Agar control		Nil

Substrate was found in all organisms sensitive to lysozyme, the largest amount being present in the non-lysable *B. subtilis*. Only traces of substrate were found in organisms unaffected by lysozyme ; agar was devoid of it.

Lysozyme Activity at Different pH Values.

The activity of lysozyme at different pH values was studied with a purified substrate preparation by estimating the amount of N-acetylhexosamine liberated in 1 c.c. samples after 24 hours' incubation with lysozyme at 37° C. The substrate was used as a 0·5 per cent. solution in the buffers and an aqueous lysozyme solution was added to bring the lysozyme concentration to 0·01 per cent. The buffers used were : pH 2–4, *M*/15 citrate ; pH 5, *M*/10 acetate ; pH 6 and 7, *M*/15 phosphate ; pH 8 and 9, *M*/10 borate. The results are given in Table V. It will be seen that lysozyme is active over a wide range from pH 2–9. In this respect it behaves similarly to testis hyaluronidase (" spreading factor ", Chain and Duthie, 1940). Another point of resemblance between the two carbohydrases, lysozyme and hyaluronidase, is their sensitivity to surface denaturation ; both are completely inactivated by mild shaking in the presence of toluene, and by chloroform without shaking. Lysozyme is most stable at pH 4.

TABLE V.—*Activity of Lysozyme at Different pH.*

Substrate incubated with lysozyme for 24 hours at 37° C.

pH.	Increase of N-acetylhexosamine as per cent. of the weight of substrate.	Increase in reducing power (in glucose equivalents) as per cent. of the weight of the substrate.
2	4·5	6·2
3	4·3	4·9
4	4·3	5·6
5	4·2	5·1
6	4·4	6·5
7	4·2	5·9
8	3·4	4·1
9	1·5	2·0

Action of Lysozyme from Different Sources on Purified Substrate.

The following preparations, showing lysozyme activity in lytic tests against *M. lysodeikticus*, were examined for power of hydrolysing the purified substrate: (1) Cat saliva, (2) human saliva, (3) human saliva boiled for 5 minutes at pH 4, (4) human tears, (5) pig duodenal secretion, (6) rabbit polymorphonuclear leucocytes.

Preparations (1) and (2) were samples used in the work of Roberts, Maegraith and Florey (1938) on the immunological properties of lysozyme from different sources. The boiled human saliva was studied because lysozyme activity of human saliva is not destroyed by boiling at pH 4, whereas its inhibitory action on *C. diphtheriae* and most of its other enzyme activity is completely destroyed by such treatment (R. Thompson, in press). The same applies to lysozyme in pig duodenal secretion ; this was collected from a duodenal fistula and kindly given to us by Mrs. M. A. Jennings and Prof.

H. W. Florey. It was precipitated with acetone and dried. A water extract was made from the dry powder by extracting 100 mg. with 2 c.c. of citrate buffer pH 4 ; this extract was boiled for five minutes, neutralized, centrifuged and the supernatant fluid used. Tears were obtained by stimulation of human eyes by a stream of air. Rabbit polymorphonuclear leucocytes were collected and kindly given to us by Dr. J. Barnes. The method for obtaining these cells was that of Hamburger (1927) ; a saline extract of the dried cells was made according to Florey (1930).

The experiments were carried out by incubating 5 c.c. of 2 per cent. purified substrate solution in phosphate buffer pH 6 for 18 hours with the following amounts of lysozyme preparations : 0·5 mg. cat saliva, 1 mg. human saliva, 0·5 c.c. " acid heated " saliva, 0·1 c.c. human tears, 0·5 c.c. extract made from pig duodenal secretion, 0·5 c.c. extract made from rabbit polymorphonuclear leucocytes. Controls with heat-inactivated lysozyme (boiled for five minutes at pH 9) were set up and the increases of N-acetylhexosamine and reducing power over the controls were determined.

All lysozyme preparations hydrolysed the purified substrate, as shown in Table VI. The lytic titres of the different lysozyme preparations correspond exactly to the enzyme activities as expressed in Table VI.

TABLE VI.—*Hydrolysis of Purified Substrate by Lysozyme Preparations from Different Sources.*

Source of lysozyme.	Increase (as per cent. of substrate) of— N-acetylhexosamine.	Reducing power (in glucose equivalents).
Cat saliva	1·5	2·4
Human saliva	0·25	0·7
,, ,, acid treated . .	3·5	..
Human tears	3·75	4·2
Pig duodenal secretion. . .	2·0	..
Rabbit polymorphonuclear leucocytes	3·5	4·2
Purified egg-white lysozyme . .	4·0	4·7

Mechanism of Lysozyme Action.

It has been shown in this work that lysozyme is an enzyme hydrolysing a water-insoluble polysaccharide contained in the bodies of lysozyme-sensitive bacteria. The polysaccharide is degraded to water-soluble products and this process can be accompanied by the complete lysis of some bacteria. In these bacteria the polysaccharide is an essential element for the maintenance of their morphological structure, apparently of greater importance than the proteins, as proteolytic enzymes do not cause lysis of these three organisms. In other lysozyme-sensitive organisms, such as *B. subtilis* and *B. anthracis*, the hydrolysis of the polysaccharide by lysozyme is followed by no sign of lysis. In these organisms the maintenance of their morphological structure does not depend exclusively on the unaltered state of the substrate for lysozyme, but this substance must still have functions of vital importance for the life of the organisms, since its hydrolysis leads to their immediate death.

Lysis of those bacteria which are capable of being dissolved by lysozyme occurs only under conditions when all the substances on which the cell structure depends are in a water-soluble state after the action of lysozyme. The state of the bacterial proteins is of especial importance in this connection. *M. lysodeikticus* will not be lysed or will be only incompletely lysed if its proteins are denatured by heat, organic solvents (alcohol, acetone, etc.) or iodine. The experiment recorded in Table VII shows that denaturation of the proteins is the cause of the resistance to lysis of heated bacteria. In this experiment samples of 1 c.c. each of a suspension of heated *M. lysodeikticus* were incubated for one hour at 37° C. with 1 c.c. of saline (control), 1 c.c. of a lysozyme solution containing 0·1 mg. of lysozyme, 1 c.c. of a solution containing 0·1 mg. of crystalline trypsin, and 1 c.c. of a solution containing both 0·1 mg. of lysozyme and 0·1 mg. of crystalline trypsin. Neither lysozyme by itself nor trypsin by itself were able to produce complete lysis, which was, however, achieved by their combined action.

TABLE VII.—*Effect of Lysozyme, Trypsin and a Combination of the Two Enzymes on Heated* M. lysodeikticus.

Boiled bacteria plus			saline	0
,,	,,	,,	trypsin	±
,,	,,	,,	lysozyme	0
,,	,,	,,	lysozyme + trypsin	C

0 = No lysis. ± = Traces of lysis. C = Complete lysis.

Lysozyme acts on the isolated substrate over the wide range from pH2 to pH 9. Lysis of *M. lysodeikticus* occurs, however, only in a much more restricted pH range, no lysis being noticeable below pH 5. The insolubility of the bacterial proteins at pH values of less than 5 is the most probable explanation of this effect.

TABLE VIII.—*Alkali Phenomenon of Nakamura. Lysis of Suspensions of* M. lysodeikticus *by Lysozyme at Different pH Values before and after addition of Alkali.*

Dilution of lysozyme.	Before addition of alkali. Lysis at pH—								After addition of alkali. Lysis at pH—							
	2.	3.	4.	5.	6.	7.	8.	9.	2.	3.	4.	5.	6.	7.	8.	9.
1 : 400	0	0	0	P	+	+	+	AC	AC	+	+	+	+	+	+	AC
1 : 800	0	0	0	P	+	+	+	0	AC	+	+	+	+	+	+	P
1 : 1600	0	0	0	P	AC	P	P	0	AC	AC	AC	AC	+	+	+	0
1 : 3200	0	0	0	P	P	0	0	0	AC	P	AC	AC	+	P	AC	0
1 : 6400	0	0	0	0	0	0	0	0	P	P	P	P	AC	P	0	0
1 : 12800	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = No lysis. P = Partial lysis. AC = Almost complete lysis. + = Complete lysis.

In spite of the absence of lysis the substrate of lysozyme present in bacteria is attacked by lysozyme; this is demonstrated by the fact that immediate and complete clearance of bacterial suspensions incubated with lysozyme at

pH values lower than 5 occurs on bringing the pH to 10–12. This phenomenon was first discovered by Nakamura (1923); its explanation is most probably the solubility of the bacterial proteins at alkaline pH. The "alkali phenomenon" of Nakamura is demonstrated by an experiment recorded in Table VIII. Serial dilutions of lysozyme were put up in a series of buffers ranging from pH 2 to 9 and a saline suspension of an 18-hour culture of *M. lysodeikticus* added to each tube. Lysis was observed after one hour's incubation at 37° C. *N*/10 soda was then added to each tube bringing the pH to about 10–12 and lysis was again observed.

SUMMARY.

(1) A high-molecular polysaccharide acted upon by purified egg-white lysozyme and by lysozyme from other sources has been isolated from the dried bodies of *M. lysodeikticus*. It is thus confirmed that lysozyme is an enzyme belonging to the class of carbohydrases.

(2) The method of isolation and purification of this polysaccharide from *M. lysodeikticus* have been described. They consist of the fractional precipitation with alcohol and acetone of solutions of *M. lysodeikticus* in cold alkaline hypochlorite or hot formamide.

(3) A simple method for the large-scale growth of bacteria on solid media has been developed.

(4) The action of lysozyme on the polysaccharide, whose properties are described, is hydrolytic; a N-acetylated amino-hexose and a substance giving all the reactions of a keto-hexose are liberated. No evidence for the presence of other sugars in the products has been obtained. The polysaccharide is hydrolysed over the range of pH 2–9.

(5) The polysaccharide, isolated from *M. lysodeikticus* as a water soluble substance, is present in bacteria in an insoluble form, possibly either in a higher polymerized state or in combination with a protein resistant to the action of pepsin.

(6) The polysaccharide acted upon by lysozyme has been found in all lysozyme-sensitive organisms investigated. The lysozyme sensitivity of some of these organisms manifests itself not by lysis, as in the case of *M. lysodeikticus*, but by the complete and irreversible inhibition of their respiration immediately after addition of lysozyme to suspensions, and by the inhibition of growth of the organisms on media containing lysozyme.

(7) Lysozyme preparations from all sources investigated have the power of hydrolysing the purified substrate.

(8) The conditions essential for lysis of bacteria by lysozyme and the mechanism of lysis are discussed.

We wish to express our thanks to Prof. H. W. Florey, at whose suggestion this work was carried out, for interest and encouragement. We are also greatly indebted to Dr. K. Meyer for providing one of us (L.E.) with the hospitality of his laboratory for certain experiments included in this paper. Our thanks are due to the Medical Research Council for grants towards

expenses, to Dr. E. P. Abraham for gifts of purified lysozyme preparations, to Lt.-Col. R. F. Bridges for helpful suggestions, to Dr. J. Barnes for a specimen of rabbit polymorphonuclear leucocytes, to A. Haynes who was in charge of the large-scale growth of *M. lysodeikticus*, and D. S. Callow for skilful assistance.

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THE ACTION OF SULPHANILAMIDE ON THE VIRUS OF LYMPHOGRANULOMA VENEREUM.

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Received for publication November 2, 1940.

Of the many theories which seek to explain chemotherapeutic action one of the most interesting is that put forward by Schulemann (1939), who pointed out that substances possessing chemotherapeutic action may interfere with the food supply of the parasites that they kill. In the case of the sulphonamide group of drugs Stacey and Schlüchterer (1939) also suggested that accessory growth factors essential for the organisms may be rendered unavailable. This suggestion has been further developed by Fildes (1940), who put forward the theory that antibacterial substances function by "interfering" with the metabolism of an "essential metabolite," an essential metabolite being defined as a substance or chemical group which takes an essential part in a chain of syntheses necessary for bacterial growth. An essential metabolite may be sythensized by the organism for which it is necessary, or it may be a growth factor which cannot be synthesized by the organism and must be present in the nutrient medium with which the organism is supplied. The interference exercised by the antibacterial substance may involve oxidation of a substance which requires to be reduced, molecular combination with the formation of an inactive product, or competition for an enzyme associated with the essential metabolite.

As a result of the work of Woods (1940) and Selbie (1940) it is believed that sulphanilamide competes for an enzyme associated with an essential metabolite, which in the case of the haemolytic streptococcus is *p*-aminobenzoic acid. Woods (1940), starting from the fact that yeast extracts contain a substance which reverses the inhibitory action of sulphanilamide on the growth of haemolytic streptococci in vitro, examined the chemical properties of the sulphanilamide "inhibitor." The behaviour of this inhibitor in in vitro growth tests suggested that it might be chemically related to sulphanilamide. *p*-Aminobenzoic acid, which chemically is very similar to sulphanilamide, has been found to be highly active as a sulphanilamide inhibitor in vitro; in addition, *p*-aminobenzoic acid is known to be synthesized by haemolytic streptococci and by *Escheria coli* when grown in nutritive media, and strong circumstantial evidence, therefore, suggests that the yeast factor may be *p*-aminobenzoic acid. Sulphanilamide is closely related chemically to *p*-aminobenzoic acid, and thus could combine with the same enzymes, but sufficiently unrelated to be devoid of essential metabolic activity. Selbie (1940) showed that in vivo *p*-aminobenzoic acid could interfere with the curative action of sulphanilamide on haemolytic streptococci, since mice inoculated

intraperitoneally with haemolytic streptococci and given mixtures of *p*-aminobenzoic acid and sulphanilamide, died as readily as did inoculated mice receiving no form of chemotherapeutic treatment.

One of the most remarkable properties of the sulphonamide groups of drugs is the fact that they inhibit the activities of a large variety of parasites, ranging from protozoa, such as plasmodia, to viruses, such as those of trachoma and lymphogranuloma venereum. In view of this wide range of chemotherapeutic activity, it appeared to be of interest to determine whether the curative activity of sulphanilamide on the virus of lymphogranuloma venereum was also inhibited by the simultaneous administration of *p*-aminobenzoic acid. Before carrying out experiments on the virus of lymphogranuloma venereum injected intracerebrally into mice it seemed, however, advisable to examine the effect of *p*-aminobenzoic acid and sulphanilamide on haemolytic streptococci injected intracerebrally in mice.

SULPHANILAMIDE AND P-AMINOBENZOIC ACID IN STREPTOCOCCAL INFECTIONS IN MICE.

In the two following experiments the Richards strain of haemolytic streptococcus was employed.

Experiment 1.

A 24-hour culture in serum broth was diluted to 10^{-6} in serum broth. Thirty-six mice were injected intracerebrally with 0·03 c.c. of this dilution while the same number received 0·1 c.c. intraperitoneally. The mice injected intracerebrally and intraperitoneally were then each divided into three groups of 12 mice. One group of intracerebrally injected and one group of intraperitoneally injected mice were kept as controls; one group of the intracerebrally injected and one group of the intraperitoneally injected mice were given 10 mg. of sulphanilamide per 20 g. of body-weight per os 20 minutes after injection of the haemolytic streptococci, and thereafter 5 mg. per 20 g. of body-weight morning and evening for three days. The total dosage of sulphanilamide was thus 40 mg. per 20 g. of body-weight. The remaining two groups of mice were similarly given 10 mg. of sulphanilamide and 10 mg. of *p*-aminobenzoic acid by mouth 20 minutes after injection and the same amount per day in divided doses morning and evening. The result of the experiment is shown in Table I, from which it will be seen that *p*-aminobenzoic acid inhibited the therapeutic action of sulphanilamide, the results, however, being less striking after intracerebral than after intraperitoneal injection, owing to the fact that of the intracerebrally injected mice treated with sulphanilamide seven died during the week following injection.

Experiment 2.

In a further experiment, therefore, the treatment with sulphanilamide and with sulphanilamide and *p*-aminobenzoic acid was given after injection of the streptococci as before but was continued for the succeeding five days. The

total dosage of sulphanilamide and also of *p*-aminobenzoic was 60 mg. per 20 g. of body-weight.

TABLE I.—*The Action of Sulphanilamide and* p-*Aminobenzoic Acid on Haemolytic Streptococci.*

Experiment 1.—Total dosages of sulphanilamide and *p*-aminobenzoic acid each 40 mg. per 20 g. of body-weight.

Mice.	Number of mice dying on each day following inoculation.										Number of survivors.
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10+.	
Intraperitoneal Injection.											
Controls	7	4	1	–	–	–	–	–	–	–	0
Sulphanilamide	–	–	–	1	1	–	–	–	–	–	10
Sulphanilamide and *p*-Aminobenzoic acid	1	6	1	–	–	1	1	2	–	–	0
Intracerebral Injection.											
Controls	3	6	–	2	–	1	–	–	–	–	0
Sulphanilamide	–	1	2	1	1	1	1	–	–	–	5
Sulphanilamide and *p*-Aminobenzoic acid	2	5	–	2	3	–	–	–	–	–	0

Experiment 2.—Total doses of sulphanilamide and *p*-aminobenzoic acid each 60 mg. per 20 g. of body-weight.

Mice.	Number of mice dying on each day following inoculation.										Number of survivors.
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10+.	
Intraperitoneal Injection.											
Controls	8	3	–	1	–	–	–	–	–	–	0
Sulphanilamide	–	–	–	1	–	–	–	–	–	–	11
Sulphanilamide and *p*-Aminobenzoic acid	2	8	1	1	–	–	–	–	–	–	0
Intracerebral Injection.											
Controls	4	5	2	1	–	–	–	–	–	–	0
Sulphanilamide	–	1	2	–	–	–	–	–	–	–	9
Sulphanilamide and *p*-Aminobenzoic acid	3	5	1	2	1	–	–	–	–	–	0

It is thus obvious that when *p*-aminobenzoic acid is fed to mice together with sulphanilamide, the latter no longer cures haemolytic streptococcal infections in mice, since the mice thus treated die almost as rapidly as the untreated control mice. This result may well be explained by the fact that *p*-aminobenzoic acid inhibits the chemotherapeutic action of sulphanilamide. An alternative explanation, however, would be that *p*-aminobenzoic acid and sulphanilamide when fed together to mice are toxic, whereas each of the compounds separately is non-toxic. In order to make certain that this latter explanation was not the correct one, the following experiment was undertaken: Thirty mice were injected intracerebrally with 0·03 c.c. of sterile broth and 30

intraperitoneally with 0·2 c.c. of sterile broth. The intracerebrally and the intraperitoneally inoculated mice were then each divided into three groups of 10. Ten of the intracerebral and 10 of the intraperitoneal mice were given daily per os 10 mg. of sulphanilamide suspended in gum acacia per 20 g. mouse ; the same numbers of mice were given 10 mg. of *p*-aminobenzoic acid suspended in gum acacia per 20 g. mouse per day, and the remaining 20 mice were similarly given 10 mg. of sulphanilamide and 10 mg. of *p*-aminobenzoic acid per 20 g. mouse. The daily dose was divided into two equal amounts and given morning and evening for six days. With the exception of one of the intracerebrally injected mice given *p*-aminobenzoic acid which died immediately after being fed, all the mice remained in good health. Sulphanilamide and *p*-aminobenzoic acid in the doses administered did not, therefore, exhibit a combined toxicity greater than that of the two compounds administered separately. It therefore appears probable that *p*-aminobenzoic acid interfered in mice with the chemotherapeutic action of sulphanilamide on the haemolytic streptococcus whether the organisms were inoculated intracerebrally or intraperitoneally.

THE EFFECT OF *p*-AMINOBENZOIC ACID ON THE ACTION OF SULPHANILAMIDE ON THE VIRUS OF LYMPHOGRANULOMA VENEREUM.

In order to determine whether the action of sulphanilamide on the virus of lymphogranuloma venereum could also be inhibited by the simultaneous administration of *p*-aminobenzoic acid, two experiments were carried out, each involving the use of 150 mice. All the mice were injected intracerebrally with 0·03 c.c. of a 10^{-1} suspension in physiological saline of mouse brain infected with the virus of lymphogranuloma venereum. Fifty mice were kept as controls, 50 mice were fed daily with 10 mg. of sulphanilamide suspended in gum acacia, and 50 mice were fed with 10 mg. of sulphanilamide and 10 mg. of *p*-aminobenzoic acid suspended in gum acacia. The results of the two experiments are shown in Table II.

TABLE II.—*The Effect of* p-*Aminobenzoic Acid and Sulphanilamide on the Virus of Lymphogranuloma Venereum in Mice.*

	Mice treated with sulphanilamide.			Mice treated with sulphanilamide and *p*-aminobenzoic acid.			Mice controls.		
	Exp. 1.	Exp. 2.	Total.	Exp. 1.	Exp. 2.	Total.	Exp. 1.	Exp. 2.	Total.
Number of mice	50	50	100	50	50	100	50	50	100
Number of mice developing symptoms	32	40	72	47	50	97	47	48	95
Average number of days from inoculation to development of symptoms	3·5	4·39	3·94	4·53	2·10	3·31	3·02	2·04	2·53
Number of mice dying	23	26	49	38	41	79	36	48	84
Average number of days from inoculation to death	5·52	4·92	5·22	5·97	4·30	5·13	4·75	3·86	4·30
Number of mice surviving without symptoms	18	10	28	3	0	3	3	2	5
Total number of survivors	27	24	51	12	9	21	14	2	16

DISCUSSION.

The inhibitory action of sulphanilamide on the virus of lymphogranuloma venereum is not as striking as on haemolytic streptococci; as a result it is not to be expected that the effect obtained with *p*-aminobenzoic acid on the virus infection would be as striking as in the case of the streptococcus. Nevertheless, from the combined result of the two experiments it is obvious that *p*-aminobenzoic acid given with sulphanilamide inhibits to a high degree the therapeutic action of sulphanilamide on the virus. If the theory that the inhibitory action of *p*-aminobenzoic acid is due to the fact that it competes with sulphanilamide for a particular enzyme be correct, it follows that *p*-aminobenzoic acid or some substance formed from *p*-aminobenzoic acid in the tissues is an essential metabolite of the virus of lymphogranuloma venereum. Whether this substance can be synthesized by the virus itself or is an essential growth factor which must be supplied by the tissues in which the virus grows is a question requiring further investigation. Since the viruses of lymphogranuloma venereum and trachoma are the only ones known to be acted upon by sulphanilamide, it is tempting to regard these viruses as the only two which require *p*-aminobenzoic acid for their metabolism. For all other viruses *p*-aminobenzoic acid would thus not be an essential metabolite. An alternative theory, however, of the failure of sulphanilamide to act on all other viruses would be that in the course of their metabolism so much *p*-aminobenzoic acid is formed that the chemotherapeutic action of sulphanilamide is completely inhibited. Experiments are at present being undertaken to determine whether this alternative theory has any validity.

SUMMARY.

The inhibition by *p*-aminobenzoic acid of the chemotherapeutic activity of sulphanilamide on haemolytic streptococci in mice is confirmed.

The inhibitory action is shown following both intracerebral and intraperitoneal inoculation of haemolytic streptococci in mice.

p-Aminobenzoic acid also inhibits the chemotherapeutic action of sulphanilamide on the virus of lymphogranuloma venereum injected intracerebrally in mice.

p-Aminobenzoic acid, or some closely allied compound formed from it in the tissues, would thus appear to be an essential metabolite for the virus of lymphogranuloma venereum.

The failure of sulphanilamide to act on other viruses, with the exception of that of trachoma, is briefly discussed in relation to this conception.

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INACTIVATION OF COBRA VENOM BY FINELY DISPERSED EMULSION.

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Received for publication November 5, 1940.

FINELY dispersed particles of oil can, under certain conditions, prevent bacterial toxins from exerting their lethal effects (Frazer and Walsh, 1934; Myers, 1934; and Oerskov and Schmidt, 1935). Further investigations have shown (Frazer and Walsh, 1939) that there is a temperature factor in this phenomenon. Thus more rapid and complete detoxication is obtained at body than at room temperature. The detoxicating mechanism is thought to be adsorption of the toxin at the oil/water interface (Frazer, 1936). Cobra venom has been found to behave similarly to the bacterial toxins when mixed with finely dispersed emulsions (Frazer and Stewart, 1938). The object of this communication is to put forward the evidence to show that the inactivation of cobra venom by finely dispersed oil-in-water emulsion is due to adsorption at the oil/water interface.

MATERIALS USED.

Cobra venom: Venom 1. Dilution for mice, 1 : 10,000 (dose 0·25 c.c.; kills adult mouse in 2 hours).

Venom 2. Dilution for rabbits, 1 : 500 (intravenous dose 0·4 c.c. causing death in 1–2 hours).

Emulsions:

S–S (Soap stabilized) 5 per cent. olive oil-in-water emulsion containing 0·2 per cent. of sodium oleate as stabilizer, pH 8·5. Maximum particle size 0·5μ. Prepared on the apparatus described by Frazer and Walsh (1933).

P–P 1. (Protein-protected) Emulsion S–S to which an equal volume of egg white solution (7 per cent. total protein) has been added.

P–P 2. Emulsion S–S to which an equal volume of plasma has been added.

A–P. (Albumin-protected) Emulsion S–S to which an equal volume of albumin (separated) has been added.

G–P. (Globulin-protected) Emulsion S–S to which an equal volume of globulin (separated) has been added.

Alkaline soap solution. 0·2 per cent. sodium oleate at pH 8·5.

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EXPERIMENTAL RESULTS.

Series 1.—*The Effect of Intraperitoneal Injection of Venom-emulsion Mixtures in Mice.*

Twelve mixtures were made up in three groups :

A. 0·3 c.c. Venom 1 + 0·75 c.c. S–S emulsion.
B. 0·3 c.c. Venom 1 + 0·75 c.c. distilled water.
C. 0·3 c.c. Venom 1 + 0·75 c.c. alkaline soap solution.

All the mixtures were made at 37° C., and maintained at this temperature for 15 minutes prior to intraperitoneal injection. All the animals died within two hours. The animals in Group A stayed relatively well for over an hour, and then quite suddenly the symptoms of intoxication appeared and the animals rapidly died. The condition of the animals in Groups B and C deteriorated from the start. Post-mortem examination revealed the presence of large white flakes of broken-out emulsion in the peritoneal cavity in Group A.

Series 2.—*The Effect of the Intravenous Injection of Venom-emulsion Mixture in Rabbits.*

Mixtures were made up similar to the first series, but using 0·4 c.c. doses of Venom 2. The mixtures were incubated at 37°C. for 15 minutes and then injected intravenously. The animals in the control Groups B and C all died in 1–1½ hours, but the animals in Group A receiving the venom-emulsion mixture showed no ill-effects. These animals continued to feed and behave quite normally throughout the experiment. Subsequently these same animals had several further lethal doses of venom combined with emulsion, and they showed no signs of intoxication.

Series 3.—*The Rate of Development of Symptoms after Intraperitoneal Injection of Venom-emulsion Mixtures.*

The action of cobra venom on the mouse is to produce a curare-like paralysis of the respiratory muscles (Kellaway, 1937). This results in a progressive slowing of the respiration from the normal rate of 200 per minute to a few gasps per minute accompanied by convulsions which immediately precede death.

By counting the respirations per minute some indication of the degree of intoxication can be obtained. Groups of mice were injected in exactly the same way as Series 1. The respirations were recorded in each mouse at regular intervals. The change in the respiration rates is shown in Fig. 1. No symptoms were apparent until the respirations fell below 150, when the animal showed a disinclination for food. Shortly after this the animal became quiet and difficult to rouse, and convulsions usually started when the respirations reached about 80 per minute.

Series 4.—*Effect of Intraperitoneal Injection of Emulsion.*

Mixtures consisting of 0·3 c.c. of distilled water and 0·75 c.c. of S–S emulsion were injected into 20 mice. These mice were killed at intervals and the material

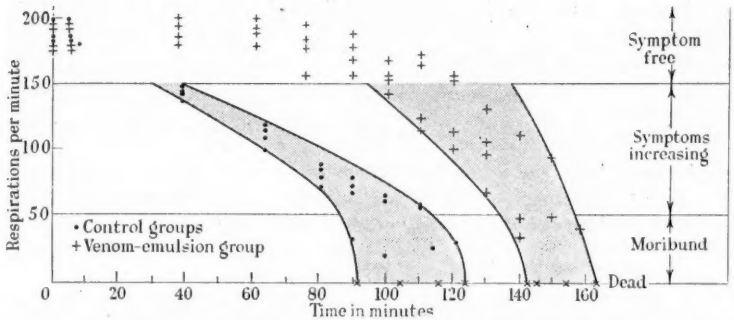

FIG. 1.—Shows comparison of symptom development rates in control mice Groups B and C compared with the venom-emulsion Group A. All animals in both groups died. The emulsion Group A were symptom-free for 90 minutes; the control Groups B and C were ill from the start.

in the peritoneal cavity was examined under dark-ground illumination. The size of the oil globules was noted and creaming began in about one hour. The rate at which the emulsion breaks out is shown in Fig. 2.

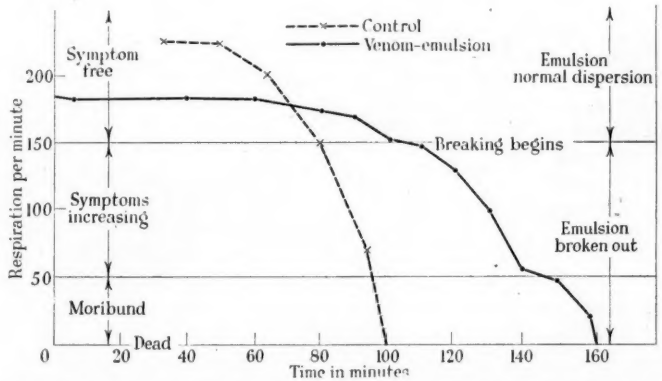

FIG. 2.—Continuous line represents the mean respiration rate of the four mice in the venom-emulsion Group A. Interrupted line represents the relative breaking rate of the emulsion when injected intraperitoneally alone. There is a close correlation in time between the initial development of symptoms in the venom-emulsion group and in the commencement of creaming of the emulsion.

Series 5.—Effect of Normal Saline on S–S Emulsion and the Protection Afforded by Protein.

A series of mixtures was made up containing varying proportions of normal saline and S–S emulsion. With a mixture of saline and emulsion in proportions of 1 : 9 creaming was evident in two hours. With proportions of 9 : 1 the emulsion was completely broken in 10 mins. The addition of an equal quantity

of egg-white or serum to the emulsion afforded complete protection, and no creaming was found after three hours with a mixture of saline and protein-protected emulsion in proportions of 9 : 1.

Series 6.—Some Further Properties of Protein-protected Emulsion.

To illustrate how very complete is the protection of the secondary protein film, the results of experiments (Frazer and Stewart, 1939) showing protection against acids and strong salt solutions are shown in Table I.

TABLE I.

	Normal saline.	Saturated NaCl.	N/5 HCl.*
1. Emulsion S–S .	++++ .	++++ .	++++
2. ,, P–P1 .	0 .	+++ .	0
3. ,, P–P2 .	0 .	+++ .	0
4. ,, A–P .	0 .	0 .	0
5. ,, G–P .	0 .	+++ .	0

++++ = Completely broken out. +++ = Gross creaming. 0 = No change.
* Care must be taken to avoid the iso-electric point of the protein.

DISCUSSION.

The results of the first series of experiments on the effect of intraperitoneal injection of venom-emulsion mixtures suggest that no protection is afforded by emulsion against cobra venom. However, there are two points of importance: firstly, the difference in the rate of development of symptoms in the animals receiving the venom-emulsion mixture and in the controls; and secondly, the presence of white flakes of creaming emulsion in the peritoneal cavities. The results of further investigation of the symptom development rate are shown in Fig. 1. In both control groups the symptoms appear very early, and many of these animals die before symptoms begin in the emulsion Group A. Once the symptoms appear in this group, the course of the intoxication is very rapid and death soon occurs. Renaud (1930) found that saturated solutions of oleate caused detoxication of cobra venom. That the 0·2 per cent. of sodium oleate, used as stabilizer for the emulsions in our experiments, is not responsible for the inactivation of the venom is shown by the rapid death of the animals in the control groups in which the diluent used is the water phase of the emulsion.

The results of Series 2 are in striking contrast to the mice experiments. Here complete detoxication is evident, and provided that the intravenous route is used, no ill-effects are seen. The dose is very large and symptoms appear a few minutes after injection, ending in death usually in about one hour.

One explanation of these findings is that the venom, being adsorbed and detoxicated by the emulsion, produces no ill-effects in the rabbit. In the peritoneal cavity of the mouse the venom-emulsion mixture is again non-toxic, and to start with no symptoms are seen. Later the emulsion begins to cream,

and as the globules increase in size, the interfacial area diminishes ; venom is thus liberated, leading to intoxication and ultimate death of the animal.

The results of the fourth series of experiments show that the emulsion does in fact cream when injected into the peritoneal cavity of the mouse. Furthermore, Fig. 2 shows the rate at which the creaming develops, and this coincides very closely with the symptom development rate curve of the emulsion group. Since the control Group B shows that the alkaline soap solution plays no part in the detoxication, the venom must be inactivated by the oil fraction. Since the venom is liberated by diminution in the area of the oil/water interface, inactivation must be due to adsorption at the interface.

The normal saline in the peritoneal transudate causes creaming of the emulsion and liberation of the venom, and yet the same concentration of saline fails to cause creaming when the emulsion is exposed to it in the blood stream. The reason for this is apparent from consideration of the results of Series 5 and 6.

In the former the addition of protein solutions to the emulsion protects the globules against the saline. Each oil globule in these emulsions is stabilized with a film of sodium oleate. The normal saline destroys the soap film, allowing the globules to coalesce. Mixtures of albumin and globulin or the separated fractions will all prevent this effect. The action is thought to be due to a secondary adsorbed protein film which lies external to the soap film. Table I illustrates the powerful protecting qualities of this protein film. Thus, acidity up to a pH of nearly 1 will not cause creaming, and strong salt solutions produce no effect on the albumin-protected emulsion. Emulsions mixed with globulin or with mixtures of albumin and globulin are not stable when strong salt solution is added. This secondary adsorbed film of protein gives to the fat particle certain properties which are characteristic of the protein involved. Chylomicrons found in the systemic blood also exhibit similar reactions (Elkes, Frazer and Stewart, 1939). Thus venom-emulsion mixtures introduced into the blood stream receive protein protection and no ill-effects follow. The mixture given intraperitoneally receives no protection, creaming occurs, and intoxication ensues.

SUMMARY.

1. A lethal dose of cobra venom is rendered innocuous by mixture with finely dispersed oil-in-water emulsion.

2. The venom-emulsion mixture is harmless intravenously, but causes death if given intraperitoneally.

3. Emulsion injected intraperitoneally creams, and the rate of creaming coincides with the rate of development of the symptoms of intoxication as shown by the respiration rate of the mouse.

4. Soap stabilized emulsion will cream if mixed with normal saline, strong salt solutions, or acids, due to interference with the stabilizing soap film. If protein is added, protection can be demonstrated. Protein-protected emulsions do not cream when normal saline or acid is added to them.

5. It is concluded that cobra venom is inactivated by adsorption at the oil/water interface of an oil-in-water emulsion. If this mixture is injected

intravenously the plasma protein affords protection to the emulsion and there are no ill-effects. If it is given intraperitoneally there is no protein protection, creaming occurs, with liberation of the venom and death of the animal.

We should like to thank Dr. C. E. van Rooyen for providing the cobra venom and the Sir Halley Stewart Trust for their generous support throughout.

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